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(54) **DNA sequence comprising a structural gene coding for xylose reductase and/or xylitol dehydrogenase.**

(57) The invention relates to a DNA sequence comprising a structural gene encoding xylose reductase and/or xylitol dehydrogenase and being capable of expressing these polypeptides in a microorganism. The invention further relates to a combination of DNA sequences, a vector, a microorganism, a method for producing xylose reductase and/or xylitol dehydrogenase and the protein xylulose reductase and/or xylitol dehydrogenase. Microorganisms, expressing the structural genes comprised by the inventive DNA sequences may be used for producing ethanol from xylulose, for producing biomass and recovering NADP⁺ from NADPH. Preferred microorganisms are S. cerevisiae and Schizosaccharomyces pombe.

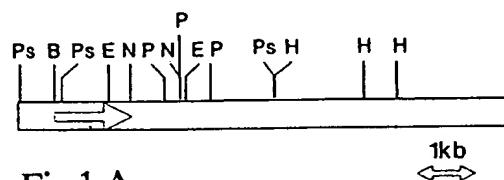


Fig.1 A

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EUROPEAN SEARCH REPORT

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- page 1 -

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.)
X	Derwent Biotechnology Abstracts, Accession no. 86-08636 & Abstr. Annu. Meet.Am.Soc.Microbiol. 1986, Abstract 0-7, BOLEN et al.: "Identification of a cDNA clone encoding aldose reductase from xylose-fermenting yeast Pachysolen tannophilus"	1,2,8, 9,16, 18,25, 29	C12N15/53 C12N9/02 C12P21/02 C12N1/14 C12P7/10
Y		35	
D,X	CURRENT GENETICS vol. 16, 1989, Berlin, DE; pages 27-33, J. HAGEDORN et al.: "Isolation and characterization of xyl mutants in a xylose-utilizing yeast, Pichia stipitis" * page 32, left-hand column, line 3 - page 33, right-hand column, line 13 *	34	
D,A		1,2	
Y	EP-A-238023 (NOVO INDUSTRI A/S) * the whole document *	35	TECHNICAL FIELDS SEARCHED (Int. Cl.)
Y	Derwent Publication Ltd., London, GB, Database WPIL, accession no. 86-123031, week 8619 & JP61063291(DAIICHI KOGYO SEIYAKU) 01.04.86	4,5,9, 16,18, 22,29	
Y	APPLIED MICROBIOLOGY AND BIOTECHNOLOGY vol. 30, no. 4, April 1989, pages 351-357, Berlin, DE; R. AMORE et al.: "The fermentation of xylose - an analysis of the expression of Bacillus and Actinoplanes xylose isomerase genes in yeast" * the whole document *	4-7,9, 16,18, 20,22, 29	
Y	Derwent Publication Limited, London, GB Database WPIL, accession no. 85-287878, Week 8546 & JP60199383(Morimoto S.), 08.10.1981	6,7,20	
A		1,4,25, 29	
The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
BERLIN		29.08.1991	GURDJIAN, D.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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CLAIMS INCURRING FEES	
<p>The present European patent application comprised at the time of filing more than ten claims.</p> <p><input type="checkbox"/> All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.</p> <p><input type="checkbox"/> Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid. namely claims:</p> <p><input type="checkbox"/> No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.</p>	
X LACK OF UNITY OF INVENTION	
<p>The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions. namely:</p> <p>1. claims: 1-3 (partly), 4,5,8-28 (partly), 29,31-35 (partly): DNA encoding xylose reductase, vector and host containing it, method for producing xylose reductase, xylose reductase, production of ethanol and biomass, process for recycling NADP+ and expression system</p> <p>2. claims: 1-3 (partly), 6,7,8-28 (partly), 30,31-35 (partly): DNA encoding xylitol dehydrogenase, host and vector containing it, method for producing xylitol dehydrogenase, xylitol dehydrogenase, production of ethanol and biomass, expression system</p>	
<p><input checked="" type="checkbox"/> All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.</p> <p><input type="checkbox"/> Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid. namely claims:</p> <p><input type="checkbox"/> None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims. namely claims:</p>	

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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	US-A-4840903 (JUNG FU WU) * abstract *	31,32	
A	GB-A-2151635 (IMPERIAL CHEMICAL INDUSTRIES PLC) * abstract *	32,33	
P,X	CURRENT GENETICS vol. 18, 1990, pages 493-500, Berlin, DE; P. KÖTTER et al.: "Isolation and characterization of the <i>Pichia stipitis</i> <i>xylitol dehydrogenase gene, XYL2</i> , and construction of a xylose utilizing <i>Saccharomyces cerevisiae</i> transformant" * the whole document *	1-32	TECHNICAL FIELDS SEARCHED (Int. Cl.)

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The microorganism has been deposited with
Deutsche Sammlung von Mikroorganismen und
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(54) DNA sequence comprising a structural gene coding for xylose reductase and/or xylitol dehydrogenase.

(57) The invention relates to a DNA sequence comprising a structural gene encoding xylose reductase and/or xylitol dehydrogenase and being capable of expressing these polypeptides in a microorganism. The invention further relates to a combination of DNA sequences, a vector, a microorganism, a method for producing xylose reductase and/or xylitol dehydrogenase and the protein xylulose reductase and/or xylitol dehydrogenase. Microorganisms, expressing the structural genes comprised by the inventive DNA sequences may be used for producing ethanol from xylulose, for producing biomass and recovering NADP⁺ from NADPH. Preferred microorganisms are S. cerevisiae and Schizosaccharomyces pombe.

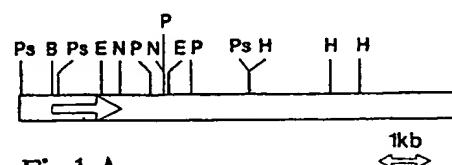


Fig.1 A

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The present invention relates to a DNA sequence, a combination of DNA sequences, a vector, a microorganism, a method for producing xylose reductase and/or xylitol dehydrogenase, xylose reductase and xylitol dehydrogenase; the invention further relates to an ethanol manufacturing process, a process for production of biomass, a process for recycling of NADP⁺ from NADPH and a method for producing a desired protein in Pichia stipitis.

D-xylose is one of the most abundant carbohydrates occurring in plant biomass and wood. In the process of cellulose production, it is formed as a waste product from hydrolysis of xylan, which is the major compound of hemicellulose. To optimize the use of renewable carbon sources, it is desirable to convert xylose into ethanol or biomass. There are several yeast species, such as Candida (Gong et al., 1981, Jeffries, 1983), Debaryomyces, Hansenula, Kluyveromyces, Metschnikowia, Pachysolen, Paecilomyces (Wu et al., 1986) and Pichia (Maleszka and Schneider 1982), which are able to utilize pentoses, including D-xylose, and D-ribose, however, only aerobically.

In general, pentoses utilized by yeasts (e.g. Pichia stipitis) must be isomerized to pentuloses in order to be phosphorylated. This isomerization occurs via a NAD(P)H linked reduction (reductase) to pentitols followed by NAD⁺-linked oxidation (dehydrogenase) of the pentitols to the corresponding D-pentuloses (Barnett, 1976). The yeast mainly used in bioethanol production, S. cerevisiae, can utilize xylulose, however, this yeast is not able to ferment pentoses (Jeffries, 1988). It cannot be excluded, that S. cerevisiae also contains genes, coding for pentose fermenting proteins which however are not expressed.

Pentose fermentation by S. cerevisiae may be possible by providing a xylose utilising pathway from a 20 xylose metabolizing organism. However, although many attempts have been undertaken to express bacterial xylose isomerase genes in S. cerevisiae, no xylose fermentation could be obtained probably due to inefficient expression of the foreign gene (Sarthy et al., 1987, Amore et al., 1989, Chan et al., 1986 & 1989).

Therefore it is a primary object of the present invention to provide genes of the enzymes involved in xylose degradation in order to be able to manipulate these genes, for example to combine these sequences 25 with suitable regulating sequences.

This object has been solved by a DNA sequence comprising a structural gene coding for xylose reductase and/or xylitol dehydrogenase and being capable of expressing said polypeptide(s) in a microorganism.

Further objects of the present invention will become apparent by the following detailed description of 30 the invention, the examples and figures.

Throughout this application various publications are referenced by the first author within parenthesis.

Full citations of these references may be found at the end of the specification as an annex. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the 35 invention described and claimed herein.

The DNA sequences according to the present invention preferably are derived from a yeast. Preferred yeast strains are selected from the genera Schwanniomyces, Saccharomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen and Paecilomyces. All of these yeast genera are known to be able to convert xylose into ethanol using xylose reductase and xylitol dehydrogenase.

A preferred genus used as a source for the DNA sequence according to the present invention is the yeast Pichia. This genus comprises several species, any of which could be applied for performing the present invention. However, the preferred species is Pichia stipitis. The present inventors used Pichia stipitis CBS5773 for isolation of the DNA sequences comprising a structural gene coding for xylose reductase and/or xylitol dehydrogenase. Pichia stipitis CBS5773 was redeposited under the Budapest Treaty on March 21, 1990 (DSM 5855).

The present inventors succeeded to isolate DNA molecules containing a sequence comprising the structural gene encoding a xylose reductase and a xylitol dehydrogenase respectively. By way of the DNA sequence, which was determined according to standard procedures, the amino acid sequence of both these 50 proteins could be determined for the first time. The complete amino acid sequences as well as the nucleotides sequences of both these proteins are shown in Figures 2A and 2B. As is known to everybody skilled in the art the proteins having the amino acid sequences as shown in Figures 2A and 2B can be encoded not only by the DNA sequences as found in Pichia stipitis CBS5773, but also by using alternative codons provided by the degeneracy of the genetic code. The invention thus is not limited to the DNA 55 sequence as shown in Figure 2, but also comprises any modification yielding the same amino acid sequences.

The DNA sequences according to the present invention may not only be obtained by applying the methods shown below, i.e., by isolating cDNA clones, which further on are used to screen a genomic

library, but also may be obtained by other methods of recombinant DNA technology from either natural DNA or cDNA or chemically synthesized DNA or by a combination of two or more of these DNAs. For example, it may be attempted to combine a chemically synthesized 5' region with a cDNA coding for the 3' region or any other combination of the three DNA sources mentioned above.

5 According to the present invention there are also provided combinations of DNA sequences, which comprise a DNA sequence as discussed above, i.e., a sequence comprising a structural gene coding for a xylose reductase and/or xylitol dehydrogenase, and in addition one or more DNA sequences capable of regulating the expression of the structural genes mentioned above in a presumptive host microorganism. DNA sequences capable of regulating the expression of structural genes are well known to those skilled in
 10 the art. For example, the DNA sequences discussed above may be combined with promoters, which are connected with the structural genes in order to provide efficient expression. Further DNA sequences capable of regulating the expression may comprise enhancers, termination sequences and polyadenylation signals. Examples for the best known kind of regulating sequences, are shown by the following examples.

In order to express the DNA sequences and/or the combination of DNA sequences according to the
 15 present invention efficiently, small modifications of the DNA sequences may be performed, as long as their capability to express a functional enzyme having the desired xylose reductase or xylitol dehydrogenase activity is retained. These modifications may include either variations of the genetic code as discussed above or furthermore small substitutions of the amino acid sequence, as well as deletions and/or insertions, which do not have any detrimental impact on the respective enzyme activity.

20 In a preferred embodiment the DNA sequence, capable of regulating the expression of the structural gene, is derived from an endogenous gene of the microorganism, in which expression of the DNA sequence is intended. Since, as will be shown below in more detail, Saccharomyces cerevisiae is one of the preferred microorganism to be used in the present invention, there are a multitude of possible regulating sequences known. Some of these well-known sequences have been used to construct expression vectors, as will be
 25 shown below in the examples. In the most preferred embodiment the combination of DNA sequences comprises inducible promoters. In this case the expression of xylose reductase and xylitol dehydrogenase can be prevented, as long as desired; expression may be started upon addition of a suitable inducer.

In the most preferred embodiments of the present invention the following Saccharomyces cerevisiae promoters are used to regulate the expression of the genes encoding xylose reductase and/or xylitol
 30 dehydrogenase: ADH1, ADH2, PDC, GAL1/10.

Depending on the choice of the respective promoter it may be possible to obtain expression levels exceeding that of natural expression of both proteins in their original host organism.

The DNA sequences as well as the combinations of the DNA sequences according to the present invention may be introduced in vector molecules. These molecules may be plasmids, which are suitable for
 35 replication in the desired host microorganism and thus should contain a functional origin of replication. Alternatively, it is also possible, to use linear DNA fragments carrying the DNA sequence or combination of DNA sequences according to the present invention or to use circular DNA molecules being devoid of a functional origin of replication. In this case the vector, which is not capable of replication, will be inserted by either homologous or nonhomologous recombination into the host chromosome.

40 Subject of the present invention are further microorganisms, which have received DNA sequences comprising the inventive DNA sequences or combinations of DNA sequences coding for xylose reductase or xylitol dehydrogenase by recombinant DNA technology.

Preferred microorganisms are selected from a group consisting of yeast of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces,
 45 Metschnikowia, Pachysolen or Paecilomyces or bacteria of the genus Zymomonas.

From these organisms the most preferred microorganisms are Saccharomyces cerevisiae and Schizosaccharomyces pombe and Zymomonas.

One of the possible applications of the genetically altered yeast strains described above is the production of biomass. Since the yeast strains having acquired the ability of expressing xylose reductase
 50 and/or xylitol dehydrogenase are maintaining good fermentation abilities, biomass can be produced most efficiently by use of these inventive yeast strains. The methods for producing biomass are the usual ones, which are known to everybody skilled in the art. The genetically manipulated yeast strains provided in compliance with this invention are also suitable for the production of ethanol. The preferred organisms for use in the production of ethanol by fermentation are the yeasts Saccharomyces cerevisiae and/or
 55 Schizosaccharomyces pombe and/or the bacterium Zymomonas.

The preferred carbohydrate in the ethanol production is xylose. Thus, strains of Saccharomyces cerevisiae and/or Schizosaccharomyces pombe and/or Zymomonas being able to ferment xylose are highly advantageous in the production of ethanol. The production of potable spirit or industrial ethanol by use of a

genetically manipulated yeast strain according to the present invention can be carried out in a manner known per se. The inventive yeast strains have the ability to ferment concentrated carbohydrate solutions, exhibit high ethanol tolerance and have the ability of producing elevated concentrations of ethanol; they have a high cell viability for repeated recycling and exhibit remarkable pH-and temperature tolerance. In the process of xylose production xylose is formed as a waste product from hydrolysis of xylan, which is the major compound of hemicellulose. Hence it is of great advantage to use xylose for the production of ethanol and/or biomass. The invention is further suitable for the production and isolation of the NAD(P)H linked xylose reductase. Due to the reduction reaction this enzyme is suitable for the delivering or recycling (from NADPH to an NADP⁺) of the corresponding coenzyme especially in bioreactors, for example for the production of amino acids.

A further subject of the present invention is a method for producing the xylose reductase and/or xylitol dehydrogenase by cultivating a microorganism according to the present invention under suitable conditions and recovering said enzyme or both of them in a manner known per se. The method thus includes the expression of a DNA sequence or a combination of DNA sequences according to the present invention in a suitable microorganism, cultivating said microorganism under appropriate conditions and isolating the enzyme.

It could be shown, that the level of expression of desired proteins in the inventive microorganisms is enhanced, if the microorganism has been selected for efficient fermentation of xylulose. Thus, it is preferred, to perform the method for reproducing one or both of the proteins using microorganisms, which have been selected accordingly.

Since the present invention provides the cloned genes and the corresponding sequences, the gene products can be overproduced in other organisms, e.g. in yeasts of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen, Paecilomyces or bacteria of the genus Zymomonas. The techniques employed for obtaining expression of the XYL1 (xylose reductase) and/or XYL2 (xylitol dehydrogenase) gene and the isolation of the active gene product are the usual ones such as promoter-fusion, transformation, integration and selection, and methods of protein isolation, known by the man skilled in the art.

Generally, said microorganisms have received the DNA sequence or combination of DNA sequences via transformation procedures. For each of the possible microorganisms, i.e. the different yeast genera and bacteria of the genus Zymomonas, there are transformation procedures known. The transformation is preferably carried out using a vector, which may be either a linear or circular DNA molecule; in addition, the method can be performed using autonomously replicating or integrative molecules as well. In the case, that the molecule is supposed to integrate into the genome of the respective host, it is preferred, to use a vector containing DNA, which is homologous to the DNA of said intended host microorganism. This measure facilitates homologous recombination.

Further subjects of the present invention are the enzymes produced according to the above described method.

The microorganisms according to the present invention may be used in ethanol manufacturing processes. Since xylose is a readily available source, which normally is considered to be waste, the ethanol manufacturing process according to the present invention provides a possibility for ethanol production of high economical and ecological interest.

The ethanol manufacturing process may be adapted for the production of alcoholic beverages or single cell protein from substrates containing free xylose, which is preferably released by xylanase and/or xylosidase activity from xylan.

According to the present invention there is further provided a method for the production of a desired protein in Pichia stipitis. According to this method a structural gene coding for a desired protein is expressed under control of the 5' regulating region of the XYL1 and/or XYL2 gene from Pichia stipitis and/or the ADH1 promoter of S. cerevisiae and/or the glucoamylase promoter from Schwanniomyces occidentalis. Out of the promoters mentioned before use of the 5' regulating regions of the XYL1 or XYL2 genes is preferred, because these promoters may be induced by adding xylose. Pichia stipitis, when used as a host organism, exhibits the great advantage of having an efficient secretion system. This facilitates an efficient expression not only of proteins, which stay inside the cell, but also of proteins, which are continuously secreted into the medium. A further advantage of the Pichia stipitis expression system is the possibility of using xylose as a substrate. Xylos is a rather inexpensive, readily available nutrient.

The invention will be discussed in detail by way of the following figures and examples.

BRIEF DESCRIPTION OF THE FIGURES:

Fig. 1

A: restriction map of the DNA fragment encoding the xylose reductase gene (XYL1)

E: EcoRI, H: HindIII, B: BamHI, N: NcoI,

P: PvuII, Ps: PstI

5 B: restriction map of the DNA fragment encoding the xylitol dehydrogenase gene (XYL2)

Ba: BamHI, B: BglII, E: EcoR1, X: XbaI, S: SalI

Fig. 2

A) Nucleotide sequence of the XYL1 structural gene including its 5'- and 3'-flanking sequences and the corresponding amino acid sequence.

10 B) Nucleotide sequence of the XYL2 structural gene including its 5'- and 3'-flanking sequences and the corresponding amino acid sequence.

Fig. 3 S. cerevisiae and S. pombe expression vector. Plasmid pRD1 contains both the xylose reductase gene and xylitol dehydrogenase gene under control of their original promoters.

15 Fig. 4 Fermentation curve of PK4 grown in YNB, 2% xylose medium. The culture was inoculated with 10⁸cells/ml from a xylose grown preculture. The figure shows xylose consumption and conversion into ethanol with a theoretical maximum yield.

Fig. 5 (1,2) Construction scheme for constructing the vector pBRPGAM. For constructing this vector, the 3.8 kb EcoRI-PvuII-fragment from pBRSWARSGAM containing the functional GAM promoter and base pairs 1 to 208 of the coding GAM sequence was ligated to the small EcoRI-PvuII-fragment of pBR322.

20 Fig. 6 (1,2) Construction scheme for constructing the vector pBRGC1. For constructing this vector, the 3.4 kb PvuII-fragment of pCT603 containing the structural gene for xylose starting with nucleotide + 122 was inserted into the PvuII site of vector pBRPGAM.

Fig. 7 (1,2) Construction scheme for constructing the vector pMPGC1-2. The 6.5 kb BamHI-PstI-fragment of pBRGC1 containing the cellulase gene under control of the GAM promoter was ligated with the large BamHI-PstI-fragment of pCJD5-1.

EXAMPLES

Materials and Methods

30

I. Microorganisms and cultivation

Yeast strains:

1. S. cerevisiae:

a) XJB3-1B (MAT α , met6, gal2) was obtained from the Yeast Genetic Stock Center (see Catalogue of the Yeast Genetic Stock Center, 6. edition, 1987).

35 b) GRF18 (MAT α , leu2-3, leu2-112, his3-11, his3-15) was obtained from G.R. Fink (DSM 3796).

c) AH22 (MAT α , can1, his4-519, leu2-3, leu2-112) was obtained from A. Hinnen (DSM 3820).

2. Schizosaccharomyces pombe (leu1-32, his5-303) (DSM 3796).

3. P. stipitis CBS5773 (DSM 5855) was obtained from Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands.

40 Yeast strains were grown at 30°C in YP medium (1% yeast extract, 2% bacto pepton) or in 0.67% Difco yeast nitrogen base (YNB) without amino acids, optionally supplemented with appropriate amino acids. Media were supplied with either 2% xylose or 2% glucose. The yeasts were transformed according to Dohmen et al. (1989).

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E. coli strains:

1. DH5 α F' (supplied by BRL company, Eggenstein, FRG)

2. HB101 (DSM 3788) (Bolivar et al., 1977).

45 E. coli strains were grown at 37°C in rich medium (LB-medium, Maniatis et al., 1982). The medium was supplemented with penicillin G (100 μ g/ml) when selecting for transformants. E. coli transformation was carried out as described by Maniatis (1982).

II. Purification of the XR and XDH proteins from P. stipitis

50 Cells were grown under induced conditions to exponential growth phase. To prepare cell-free extracts cells were harvested by centrifugation and were broken with glass beads in a Braun homogenizer using 0.1 M Tris-HCl buffer (pH 7.0). The supernatant obtained following 1 h centrifugation of the crude extract (150000 x g) was loaded on an affinity chromatography column (Affi-Gel Blue, 60x50 mm) preequilibrated with 5 mM NaPO₄ buffer (pH 6.8) and eluted with 1.5 mM NAD. The fractions containing XR and XDH activity were pooled and dialysed against 20 mM Tris-HCl (pH 7.5). The dialysate was subsequently applied to a DEAE-Sephadex anion exchange column preequilibrated with 20 mM Tris-HCl

(pH 7.5). Proteins were eluted with a linear gradient (20-250 mM Tris-HCl, pH 7.5). Fractions containing the highest activity were pooled, concentrated and loaded on a SDS-PAA-gel. After running the gel was stained with 0.1 M KCl and the XR- and XDH-proteinbands were cut out, both proteins were separately eluted from the polyacrylamide gel by dialysis using 20 mM NaPO₄ (pH 8.0), 0.1% SDS; subsequently the dialysate was concentrated. All buffers contained 0.2 mM DTT (Dithiothreitol) and 0.4 mM PMSF (Phenylmethansulfonylfluoride).

5 III. Preparation of antisera

Mice were given intraperitoneal injections of 2-5 µg protein in Freund complete adjuvant. Two weeks later the same amount of protein in Freund incomplete adjuvant was injected; a third injection was administered another 2 weeks later omitting Freund adjuvant. Antiserum was harvested six weeks after the first injection.

10 IV. Immunoscreening

Antisera raised in mice against purified *P. stipitis* xylose reductase (XR) and xylitol dehydrogenase (XDH) protein, respectively, were used for screening the cDNA library following the procedure of Huynh et al. (1985). The antisera were diluted 10.000-fold. Bound antibodies were visualized using an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antibody, followed by a colour development reaction with the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in combination with nitro blue tetrazolium (NBT).

15 V. Isolation of RNA

All procedures were carried out at 0 to 4°C, if not indicated otherwise. All solutions and materials were sterilized if possible. *P. stipitis* cells were grown to midexponential phase in the presence of xylose. Yeast cells were harvested by centrifugation, washed twice with buffer 1 (20 mM NaCl, 10 mM MgCl₂, 100 mM Tris-HCl, pH 7.6) and suspended in the same buffer (1.25 ml/g cells). 1/10 volume phenol, 200 µg/ml heparin, 100 µg/ml cycloheximid and 0.4% SDS were added. Disruption of the cells was carried out by shaking with glass beads (0.45 - 0.5 mm) in a ratio of glass beads to suspension of 1:1 (v/v) in a Braun homogenizer (Braun, Melsungen). Two volumes of buffer 2 (buffer 1 containing 100 µg/ml heparin, 50 µg/ml cycloheximid, 2% SDS) were added to the homogenate, cell debris were removed by centrifugation (10000 x g, 10 min). The solution was extracted three to five times with phenol/chloroform (1:1), once with chloroform/ isoamylalcohol (24:1). The nucleic acid was precipitated by incubating the aqueous phase with 2,5 volume of ethanol in the presence of 0.2 M NaCl over night at -20°C. The precipitate was solubilized in buffer 3 (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5); SDS and LiCl were added to a final concentration of 0.1% and 4 M, respectively. The RNA was precipitated over night at +4°C. The pellet was washed twice with 70% ethanol and suspended in sterilized H₂O before use. RNA was stored at -70°C as an ethanol precipitate.

20 VI. Enzyme assays

Activities of xylose reductase (EC. 1.1.1.21) and xylitol dehydrogenase (EC. 1.1.1.9) were measured as described by Bruinenberg et al. (1983). Protein was determined with the micro biuret method according to Zamenhoff (1957) using bovine serum albumin as standard.

25 VII. Gelelectrophoresis

SDS gelelectrophoresis was carried out in 10% PAA according to Laemmli (1970).

30 VIII. Immunoblotting

Detection of antigenic proteins was carried out as described by Towbin et al. (1979) using the antisera obtained from mice. The proteins were transferred to a polyvinylidene difluoride microporous membran (Millipore, Immobilon PVDF) and were visualized by a phosphatase-coupled colour reaction (Blake et al., 1984). Alkaline phosphatase conjugated to goat anti-mouse IgG was obtained from Jackson Immunoresearch Lab. (Avondale, USA).

35 IX. DNA-sequence analysis

XYL1 and *XYL2* genomic DNA as well as the respective cDNAs were subcloned in pT7T3-18U (Pharmacia). Fragments obtained by partial digestion using Exonuclease III (Henikoff, 1984) were analysed and sequencing was carried out by the dideoxy method of Sanger et al. (1977) using the T7-SequencingTMkit (Pharmacia). Both strands were completely determined by obtaining overlapping sequences at every junction.

40 X. Construction of a *P.stipitis* CBS 5773 (DSM 5855) cDNA library

Total RNA was extracted according to the method described above. Poly (A)⁺-RNA was prepared by chromatography on an oligo(dT)-c IIulos column using essentially the method described by Maniatis et al. (1982). A cDNA library in λgt11 was prepared by the method of Gubler and Hoffman (1983) using a cDNA synthesis kit (Pharmacia) and in vitro packaging of the recombinant λgt11-DNA according to Hohn and Murray (1974) using the in vitro packaging kit supplied by Boehringer, Mannheim (FRG).

XI. Preparation of crud extracts

Cells were grown to late exponential growth phase and washed twice in buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM β -mercapto ethanol). Cells were broken in an Braun homogenizer with an equal volume of glass beads. The supernatant resulting from 5 min centrifugation at 10000 g was used in enzyme assays. Extracts for Western blot analysis were boiled in 1% SDS, 5% β -mercapto ethanol, 10 mM potassium phosphate pH 7.0 and 10% glycerol.

EXAMPLE 1:

Isolation of the xylose reductase (XYL1) and xylitol dehydrogenase (XYL2) genes.

A λ gt11 cDNA library constructed from poly (A)⁺-RNA of *P. stipitis* was screened with mouse polyclonal antibodies raised against the purified xylose reductase (XR) and xylitol dehydrogenase (XDH) proteins, respectively. Among 110.000 recombinant clones of the amplified cDNA library containing about 55.000 primary clones, seven identical XYL1 clones and three identical XYL2 clones were identified and purified. The analysis of the insert size revealed that the XYL1 clones contain two EcoR1 fragments (0.6 kb and 0.4 kb), whereas the XYL2 clones contain a single 0.55 kb EcoR1 fragment. The respective EcoR1 fragments of the λ gt11 clones were subcloned into the single EcoR1 site of plasmid pT7T3-18U (Pharmacia) resulting in plasmids pXRa (containing the 0.4 kb EcoR1 fragment of the XYL1 clone), pXRb (containing the 0.6 kb EcoR1 fragment of the XYL1 clone) and pXDH (containing the 0.55 kb EcoR1 fragment of the XYL2 clone).

These plasmids were used as a radioactive probe to screen a *P. stipitis* genomic library, which was constructed by ligation of partial Sau3A digested *P. stipitis* DNA into the single BamH1 site of the *S. cerevisiae* - *E. coli* shuttle vector YEp13 (Broach et al., 1979) resulting in about 60.000 independent clones after transformation of *E. coli* HB101.

Two plasmids, namely pR1 and pD1 could be isolated and were used for transformation of *S. cerevisiae* GRF18. XR activity could be detected in the crude extracts of the transformants containing pR1, whereas transformants carrying pD1 yielded crude extracts exhibiting XDH activity. In a mitotic stability test (Beggs 1978) the LEU2 marker and the XR or XDH gene cosegregated, indicating that pR1 and pD1 harbour the functional XYL1 (xylose reductase) and XYL2 (xylitol dehydrogenase) gene, respectively.

The plasmids pR1 and pD1 were subjected to restriction enzyme analysis yielding the map of restriction sites of the XYL1 (Fig. 1A) and XYL2 (Fig. 1B) genes, respectively.

Further subcloning experiments revealed that the XYL1 gene is encoded within a 2.04 kb BamHI genomic fragment. One of the BamHI sites is not present in the original plasmid pR1. It must have been generated during subcloning. The XYL2 gene is encoded within a 1.95 kb BamHI-XbaI fragment. The 2.04 BamHI fragment and the 1.95 kb BamHI-XbaI fragment were subcloned into the multiple cloning site of pT7T3-18U resulting in pR2 and pD2, respectively, and subjected to DNA sequence analysis. The DNA sequence of the structural gene and of the 5' and 3' non-coding region of the XYL1 and the XYL2 gene is depicted in Fig. 2A and Fig. 2B, respectively.

The DNA sequence of the XYL1 gene contains an open reading frames of 954 bp (318 amino acids) whereas that of the XYL2 gene comprises an ORE of 1089 bp (363 amino acids).

The amino acids deduced from the open reading frames are shown in Fig. 2A and Fig. 2B. The sequences correspond to an XR polypeptide and an XDH polypeptide with a calculated molecular weight of 35922 and 38526 D, respectively.

EXAMPLE 2

Expression of both the xylose reductase and xylitol dehydrogenase gene in *S.cerevisiae*.

Saccharomyces cerevisiae was contrtransformed with pR1 and pD1. The highest measurable activities of XR and XDH in *S. cerevisiae* transformed accordingly correspond to 50% of the activities of both enzymes measurable in *P. stipitis* crude extracts. In *S. cerevisiae* the genes were expressed in YNB medium containing 2% glucose as a sole carbon source, whereas in *P. stipitis* expression of both genes is repressed by glucose and induced by xylose. Taking into account the copy number of 10 of YEp13 in *S. cerevisiae* and assuming a gene dosage dependent expression one can conclude that the Pichia promoters are 20 times less efficient in *S. cerevisiae* than in *P. stipitis*.

Furthermore, a plasmid harbouring both the XYL1 and XYL2 gene including their original Pichia promoters was constructed (Fig. 3). This plasmid pRD1 was used to transform strain GRF18 by selection on leucine resulting in the transformant PK1. However, expression was not improved compared to cotransformation with separate plasmids.

EXAMPLE 3

Construction of an integrative vector containing the XYL2 gene under control of different promoters

Different expression vectors using different promoters for integration and gene expression in *S. cerevisiae* were constructed. For example the *XYL2* gene was fused to the *ADH1* promoter followed by homologous integration into the *HIS3* locus of *S. cerevisiae*. The strategy employed was as follows: The 1.5 kb *Xba*I/ *Eco*R1 fragment containing the xylitol dehydrogenase gene *XYL2* was inserted into the multiple cloning site of pT7T3-18U (Pharmacia) resulting in plasmid pXDH. To eliminate the promoter region of the *XYL2* gene this plasmid was linearized with *Xba*I (restriction site 318 bp upstream of the initiator ATG codon) and with *Pst*I to protect the 3' end of the plasmid DNA. The linear plasmid was treated with exonuclease III and subsequently with S1 nuclease to remove the DNA between the *Xba*I site and the *XYL2* structural gene. The deleted DNA molecules were recircularized, cloned in *E. coli* and the extent of deletion was determined by dideoxy sequencing. In one of the modified pXDH plasmids the 5' untranslated region and the four N-terminal amino acids were deleted. However, a new inframe ATG initiation codon was created due to the *Sph*I site from the multiple cloning site. A *Bam*HI linker was inserted into the *Hind*III site of the multiple cloning site. Subsequently, a 1.5 kb *Bam*HI fragment carrying the *XYL2* gene could be subcloned into vector pT7T3-18U resulting in additional restriction sites in front of the ATG initiation codon. The newly created 5' region is as follows: ATE CCT TGG TGT...(deletion of original amino acid 2,3 and 4).

To complete the 3' untranslated region of the *XYL2* gene a 440 bp *Eco*R1 fragment, was inserted into the single *Eco*R1 site of the 1.5 kb fragment subcloned in pT7T3-18U. This 440 bp fragment was obtained by subcloning the 440 bp *Eco*R1-*Bam*HI fragments (see Fig. 1B) into another pT7T3-18U, removing the *Bam*HI site by cutting with *Bam*HI and subsequent filling-in with Klenow polymerase. The 3' untranslated region could thus be isolated as 440 bp *Eco*R1 fragment. In the single *Bam*HI site arranged near the 5' terminus of the *XYL2* gene, which is provided by the polylinker region, the 1.8 kb *Bam*HI fragment harbouring the *S. cerevisiae* *His3* gene derived from plasmid YEp6 (Struhl et al. 1979) was inserted. To remove one of the two *Bam*HI sites the resultant plasmid was cut with *Sall* and *Xhol* and subsequently recircularized. The resulting plasmid pXDH-HIS3 contains one suitable *Bam*HI site in front of the ATG initiation codon in which the 1.5 kb *Bam*HI fragment, containing the *ADH1*-promoter (Ammerer, 1983) of *S. cerevisiae* can be inserted.

Since this plasmid does not contain any autonomous replicating sequence for *S. cerevisiae* this plasmid can be used for homologous integration (Orr-Weaver et al. 1981) into the *HIS3* locus of any *S. cerevisiae* strain.

In our integration experiments we used a mutagenized XJB3-1B strain called PUA6-1, which was isolated according the protocol of Porep, (1987) and Ciriacy, (1986). The resulting integrant PK2 is expressing the *XYL2* gene under control of the *ADH1* promoter leading to an active gene product.

EXAMPLE 4

Construction of *S.cerevisiae* and *S.pombe* integrants expressing both the *XYL1* and *XYL2* gene.

To eliminate the promoter region of the *XYL1* gene plasmid pR2 containing the *XYL1* gene on a 2,04 kb *Bam*HI fragment was linearised with *Xba*I (restriction site 362 bp upstream of the translation initiation ATG codon) and cleaved with *Sph*I to protect the 3' end of the plasmid DNA. The linear plasmid was treated with exonuclease III and subsequently with S1 nuclease to remove the DNA between the *Xba*I site and the *XYL1* structural gene. The deleted DNA molecules were recircularized, cloned in *E. coli* and the extent of deletion was determined by dideoxy sequencing. In one of the modified pR2 plasmids the 5' untranslated region was exactly deleted.

The structural gene was subcloned as a *Hind*III-*Bam*HI fragment into the corresponding sites of Ylp366 (Myers et al. 1986). In addition the *ADH1* promoter was subcloned into the *Hind*III site by blunt end ligation resulting in plasmid pXR-LEU2. Since this plasmid does not contain any autonomous replicating sequence for *S. cerevisiae* this plasmid can be used for homologous integration (Orr-Weaver et al. 1981) into the *LEU2* locus of any *S. cerevisiae* strain, e.g. strain PK2. The resulting integrant PK3 is expressing both the *XYL1* and *XYL2* genes under control of the *ADH1* promoter leading to active gene products. For expression studies in *Schizosaccharomyces*, *S. pombe* was transformed with both plasmids pXDH-HIS3 and pXR-LEU2 selecting for histidine and leucine. After extensive screening of the transformants for growth on xylose one transformant called AS1 could be isolated expressing both the *XYL1* and *XYL2* gene under control of the *ADH1* promoters.

In the same manner other *S. cerevisiae* promoters, e.g. pyruvate decarboxylase (PDC) promoter (Kellermann & Hollenberg, 1988), alcoholdehydrogenas 2 (ADH2) promoter (Russell et al., 1983) or th galactokinase (GAL1/10) promoter from plasmid pBM272, which is derived from plasmid pBM150 (Johnston and Davis, 1984) by introducing a *Hind*III site immediately following the *Bam*HI site, led to expression of active *XYL1* and *XYL2* gene product in *S. cerevisiae*.

In another set of experiments two suitable restriction sites *Bam*HI (position -9) and *Sall* (position - 15)

were introduced just in front of the XYL1 and XYL2 genes.

XYL1:5'atcttttctaGTCGACGGATCCAAGATGCCTTCTATT

...TAA terminator3'

XYL2:5'cccctaactGTCGACGGATCCAAGATGACTGCTAAC

...TAA terminator3'

These modifications were introduced by site directed mutagenesis of the 5' region using the site directed mutagenesis kit supplied by Amersham according to the instructions of the manufacturer. These restriction sites offer the possibility to fuse any promoter just in front of the ATG initiation codon. In addition the gene under control of a desired promoter can be isolated as a well defined fragment for insertion into sequences suitable for homologous integration.

For industrial or commercial purposes it is desirable to construct stable production strains of *S. cerevisiae* and/or *S. pombe*. Therefore both genes under control of the constitutive ADH1 promoter were integrated without any bacterial sequence into the chromosome of *S. cerevisiae* strain PUA6-1 via homologous integration (Orr-Weaver et al. 1981). Integration into the HO homothallism gene (Russel et al. 1986), ARS-sequence (Stinchcomb et al., 1978) or into the ADH4 gene (Paquin et al., 1986) by cotransformation with pJW6 (Fogel and Welch, 1982) is preferred resulting in strains PK3(HO), PK3(ARS) and PK3(ADH4). In the case of *S. pombe* the integration mainly occurs via illegitimate recombination. Hence only a few of the *S. pombe* integrants exhibit XR and XDH activities and have the same fermentation and growth properties as the wild type.

The *S. cerevisiae* integrants PK3, PK3(HO), PK3(ARS) and PK3(ADH4) may be improved for efficient assimilation of xylulose.

EXAMPLE 5

Isolation of a *S.cerevisiae* mutant efficiently assimilating xylulose.

S. cerevisiae strain XJB3-1B grows slowly on media containing xylulose as a sole carbon source (doubling time 10 hours). According to a protocol described by Porep (Porep, 1987) a mutant, PUA3, was isolated, which utilized xylulose more efficiently than wild type *S. cerevisiae* strains, resulting in a doubling time of approximately four hours for growth on xylulose as a sole carbon source.

Mutant strain PUA3 also converts xylulose into ethanol in the absence of respiration (Porep, 1987). In order to obtain the PUA genotype in combination with an auxiliary marker (LEU2) useful in yeast transformation, strain PUA3 was crossed to AH22 (leu2 his4). From a sporulating culture of the AH22xPUA3 diploid meiotic spore progenies were isolated which were leu2 and had the ability of efficient xylulose-utilization as observed in the original mutant, PUA3. In an analogous experiment the PUA genotype was combined with leu2 and his3 auxiliary markers by crossing strain GRF18 and PUA strain and subsequent meiotic spore isolation. This resulted in strain PUA6-1 which was PUA leu2 his3.

EXAMPLE 6

Isolation of a *S.cerevisiae* mutant efficiently converting xylose into ethanol.

Strain PUA6-1 containing the XYL1 and XYL2 genes chromosomally integrated (See Examples 3 and 4) was able to grow on xylose as a sole carbon source whereas the untransformed PUA6-1 strain was completely negative on YNB xylose media. Doubling time of the transformant strain PK3 was 4 hours on YNB 1% xylose (for comparison, doubling time on YNB 1% glucose: 2 hours). Since ethanol production was inefficient in this strain when grown on xylose and no xylose growth was observed in the absence of respiration a mutant strain with improved capability in converting xylose to ethanol was selected as follows: 10⁸ PK3 cells were mutagenized with UV (254 nm) using conditions allowing 20% to 40% of the cells survival. The surviving cells were grown for approximately 30 generations in YNB 2% xylose liquid media. After plating on xylose solid media isolates were obtained which grow significantly faster than the parent strain PK3. One isolate was further propagated and used for selection of a mutant able to grow on YNB 2% xylose plates supplemented with 2 mg/l antimycin A in order to block respiratory metabolism. This procedure yielded a mutant (PK4) which was able to convert xylose significantly more efficiently to ethanol than the original transformant strain PK3. A typical xylose fermentation protocol is depicted in Fig. 4. The ethanol yield was approximately 40% of the initial xylose. This yield corresponds to approximately 80% of the theoretical maximum yield of ethanol from xylose conversion.

EXAMPLE 7

Expression of heterologous genes in *Pichiastipitis*

Following UV mutagenesis of *Pichiastipitis* strain CBS 5773 (DSM 5855) a trp5 mutant was isolated. The trp5 mutation was identified by examining indol accumulation according to Hagedorn and Ciriacy (Hagedorn and Ciriacy, 1989).

For expression in *Pichiastipitis* plasmids were constructed which contain a replicon from *Schwanomyces occidentalis* (SwARS1), the TRP5-gene from *S. cerevisiae* (Dohmen et al., 1989) as a selective

marker and in addition a glucoamylase(GAM)-cellulase (*celD*) gene fusion under control of the glucoamylase promoter. In a first step the 3.8 kb EcoRI-PvuII-fragment from plasmid pBRSwARSGAM (Fig. 5, described in EP 89 107 780) was isolated and inserted into the 2296 bp EcoRI-PvuII-fragment from pBR322 carrying the ampicillin resistance gene and the bacterial origin of replication, resulting in plasmid pBRGAM (Fig. 5). In addition to pBR322 derived sequences this plasmid carries 3.6 kb derived from the 5' noncoding region of the glucoamylase gene from *Schwanniomyces occidentalis* and nucleotides 1 to 208 coding for the N-terminal part including the signal sequence of the glucoamylase. Subsequently, a 3.4 kb PvuII-fragment derived from plasmid pCT603 (Joliff et al., 1986) containing the coding region of the *celD*-genes from *Clostridium thermocellum* with the exception of 120 bp (corresponding to 40 amino acids) starting with the 5' terminus of the coding region was inserted into the PvuII site of the pBRGAM resulting in pBRGC1 (Fig. 6). For construction of a *P. stipitis* expression vector plasmid pCJD5-1 (EP 87 110 370.1) was cleaved with BamHI/PstI and ligated with a 6.5 kb BamHI-PstI-fragment from pBRGC1. The resulting plasmid was termed pMPGC1-2 (Fig. 7). The above described *P. stipitis* mutant trp5 was transformed with pMPGC1-2 and the transformants were identified by their capability to grow on medium free of tryptophan (tryptophan prototrophy). Transformants were examined for cellulase activity using the congo red assay (Teather & Wood, 1982). The transformants constitutively produce active cellulase (endoglucanase D) of *Clostridium thermocellum*, which is secreted into the media, indicating, that the promoter and the signal sequence encoded by the glucoamylase gene may control expression of a heterologous gene and secretion of the gene product into the medium.

Subsequently plasmid pMPGC1-2 was modified in order to substitute the glucoamylase promoter either by the *S. cerevisiae* *ADH1*-promoter or the inventive 5' regions of the *XYL1* or *XYL2* gene, respectively. It could be shown, that the expression under control of the *XYL1* or *XYL2* promoter region may be induced by xylulose, while being repressed by glucose.

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Claims

1. A DNA sequence, characterized in that said DNA sequence comprises a structural gene coding for a xylose reductase and/or xylitol dehydrogenase, and is capable of expressing said polypeptide(s) in a microorganism.
2. The DNA sequence according to claim 1, characterized in that said DNA sequence is derived from a yeast, preferably from a yeast selected from a group consisting of the genera Schwanniomyces, Saccharomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, and Pachysolen.
3. The DNA sequence according to claim 2, characterized in that the yeast is Pichia stipitis, preferably Pichia stipitis CBS 5773 (DSM 5855).
4. The DNA sequence according to any of claims 1 to 3, characterized in that said sequence comprises the structural gene encoding a xylose reductase having the following amino acid sequence:

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	M	P	S	I	K	L	N	S	G	Y	10
5											
	D	M	P	A	V	G	F	G	C	W	20
10	K	V	D	V	D	T	C	S	E	Q	30
15	I	Y	R	A	I	K	T	G	Y	R	40
20	L	F	D	G	A	E	D	Y	A	N	50
	E	K	L	V	G	A	G	V	K	K	60
25											
	A	I	D	E	G	I	V	K	R	E	70
30	D	L	F	L	T	S	K	L	W	N	80
35	N	Y	H	H	P	D	N	V	E	K	90
40	A	L	N	R	T	L	S	D	L	Q	100
	V	D	Y	V	D	L	F	L	I	H	110
45											
	F	P	V	T	F	K	F	V	P	L	120
50	E	E	K	Y	P	P	G	F	Y	C	130
55	G	K	G	D	N	F	D	Y	E	D	140

	V	P	I	L	E	T	W	K	A	L	150
5											
	E	K	L	V	K	A	G	K	I	R	160
10											
	S	I	G	V	S	N	F	P	G	A	170
15											
	L	L	L	D	L	L	R	G	A	T	180
20											
	I	K	P	S	V	L	Q	V	E	H	190
25											
	H	P	Y	L	Q	Q	P	R	L	I	200
30											
	E	F	A	Q	S	R	G	I	A	V	210
35											
	T	A	Y	S	S	F	G	P	Q	S	220
40											
	F	V	E	L	N	Q	G	R	A	L	230
45											
	N	T	S	P	L	F	E	N	E	T	240
50											
	I	K	A	I	A	A	K	H	G	K	250
55											
	S	P	A	Q	V	L	L	R	W	S	260
	S	Q	R	G	I	A	I	I	P	K	270
	S	N	T	V	P	R	L	L	E	N	280

5. The DNA sequence according to claim 4, comprising the following nucleotide sequence:

25

30

35

40

45

50

55

-350
GGATCCACAGACACTAATTGGTTCTA

5

-310
CATTATTCGTGTTCAGACACAAACCCCAGC

10

-290
GTTGGCGGTTCTGTCTGCCTCCAGC

15

-250
ACCTTCTTGCTCAACCCCCAGAAGGTGCACA

20

-230
CTGCAGACACACATAACATAACGAGAACCTGG

25

-190
AACAAATATCGGTGTCGGTGACCGAAATGT

30

-170
GCAAACCCAGACACGACTAATAAACCTGGC

35

-130
AGCTCCAATACCGCCGACAACAGGTGAGGT

40

-110
GACCGATGGGGTGCCAATTAATGTCTGAAA

45

-70
ATTGGGGTATATAATATGGCGATTCTCCG

50

-50
GAGAATTTTCAGTTCTTTCTTCATTTCTC

55

-10
CAGTATTCTTCTACAACTATACTACA

50 10 30
ATGCCTTCTATTAAGTTGAACCTCTGGTTAC

55 50
GACATGCCAGCCGTCGGTTCTGGCTGTTGG

5 70 90
AAAGTCGACGTGACACACCTGTTCTGAACAG
10 110
ATCTACCGTGCTATCAAGACCGGGTACAGA
15 130 150
TTGTTCGACGGTGCCGAAGATTACGCCAAC
20 170
GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG
25 190 210
GCCATTGACGAAGGTATCGTCAAGCGTGAA
30 230
GACTTGTTCCTTACCTCCAAGTTGTGGAAC
35 250 270
AACTACCACCAACCCAGACAAACGTCGAAAAG
40 290
GCCTTGAACAGAACCCCTTCTGACTTGCAA
45 310 330
GTTGACTACGTTGACTTGTCTTGATCCAC
50 350
TTCCCAGTCACCTCAAGTTGTTCCATTAA
55 370 390
GAAGAAAAGTACCCACCAGGATTCTACTGT
60 410
GGTAAGGGTGACAACCTCGACTACGAAGAT
65 430 450
GTTCCAATTAGAGACCTGGAAAGGCTCTT
70 470
GAAAAGTTGGTCAAGGCCGGTAAGATCAGA

490 510
TCTATCGGTGTTCTAACCTCCCAGGTGCT
 5
 530
TTGCTCTTGGACTTGTTGAGAGGTGCTACC
 10 550 570
ATCAAGCCATCTGTCTTGCAAGTTGAACAC
 15 590
CACCCATACTTGCAACAAACCAAGATTGATC
 20 610 630
GAATTCGCTCAATCCCGTGGTATTGCTGTC
 25 650
ACCGCTTACTCTTCGTTCGGTCCCTCAATCT
 30 670 690
TTCGTTGAATTGAACCAAGGTAGAGCTTTG
 35 710
AACACTTCTCCATTGTTCGAGAACGAAACT
 40 730 750
ATCAAGGCTATCGCTGCTAACGCACGGTAAG
 45 770
TCTCCAGCTCAAGTCTTGTTGAGATGGTCT
 50 790 810
TCCCCAAAGAGGCATTGCCATCATTCCTAAAG
 55 830
TCCAACACTGTCCCAAGATTGTTGGAAAAC
 50 850 870
AAGGACGTCAACAGCTCGACTTGGACGAA
 55 890
CAAGATTCGCTGACATTGCCAAGTTGGAC

910 930
ATCAACTTGAGATTCAACGACCCATGGGAC

5
 950
TGGGACAAGATT CCT ATCTT CGT CTAAGAA

10 990
GGTTGCTTTATAGAGAGGAAATAAAACCTA

15 1010
ATATAACATTGATTGTACATTAAAATTGAA

20 1030 1050
TATTGTAGCTAGCAGATT CGGAA ATT TAAA

25 1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

30 1090 1110
TCTCTATGTACATACACGTTGAAGATAGCA

35 1130
GTACAGTAGACATCAAGTCTACAGATCATT

40 1150 1170
AAACATATCTTAAATTGTAGAAA ACTATAA

45 1190
ACTTTCAATTCAAACCATGTCTGCCAAGG

50 1210 1230
AATCAAATGAGATTTTTCGCAGCCAAAC

55 1250
TTGAATCCAAAAATAAAAAACGT CATTGTC

50 1270 1290
TGAAACAACTCTATCTTATCTTCACCTCA

55 1310
TCAATT CATTGCATATCAT AAAAGCCTCCG

1330 1350
ATAGCATACAAAACCTACTTCTGCATCATAT

5 1370
CTAAATCATAGTGCCATATTCAAGTAACAAT

10 1390 1410
ACCGGTAAGAAACCTCTATTTTTTAGTCT

15 1430
GCCTTAACGAGATGCAGATCGATGCAACGT

20 1450 1470
AAGATCAAACCCCTCCAGTTGTACAGTCAG

25 1490
TCATATAGTGAACACCGTACAATATGGTAT

30 1510 1530
CTACGTTCAAATAGACTCCAATACAGCTGG

35 1550
TCTGCCAAGTTGAGCAACTTTAATTAGA

40 1570 1590
GACAAAGTCGTCTCTGTTGATGTAGGCACC

45 1610
ACACATTCTTCTCTGCCGTGAACCTGT

50 1630 1650
TCTGGAGTGGAAACATCTCCAGTTGTCAAA

55 1670
TATCAAACACTGACCAGGCTTCAACTGGTA

1690
GAAGATTCGTTTGGGATC

6. The DNA sequence according to any of claims 1 to 3, characterized in that said sequence comprises the structural gene encoding a xylitol dehydrogenase having the following amino acid sequence:

5

10

15

20

25

30

35

40

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50

55

	M	T	A	N	P	S	L	V	L	N	10
5											
	K	I	D	D	I	S	F	E	T	Y	20
10											
	D	A	P	E	I	S	E	P	T	D	30
15											
	V	L	V	Q	V	K	K	T	G	I	40
20											
	C	G	S	D	I	H	F	Y	A	H	50
25											
	G	R	I	G	N	F	V	L	T	K	60
30											
	P	M	V	L	G	H	E	S	A	G	70
35											
	T	V	V	Q	V	G	K	G	V	T	80
40											
	S	L	K	V	G	D	N	V	A	I	90
45											
	E	P	G	I	P	S	R	F	S	D	100
50											
	E	Y	K	S	G	H	Y	N	L	C	110
55											
	P	H	M	A	F	A	A	T	P	N	120
	S	K	E	G	E	P	N	P	P	G	130

	T	L	C	K	Y	F	K	S	P	E	140
5	D	F	L	V	K	L	P	D	H	V	150
10	S	L	E	L	G	A	L	V	E	P	160
15	L	S	V	G	V	H	A	S	K	L	170
20	G	S	V	A	F	G	D	Y	V	A	180
25	V	F	G	A	G	P	V	G	L	L	190
30	A	A	A	V	A	K	T	F	G	A	200
35	K	G	V	I	V	V	D	I	F	D	210
40	N	K	L	K	M	A	K	D	I	G	220
45	A	A	T	H	T	F	N	S	K	T	230
50	G	G	S	E	E	L	I	K	A	F	240
55	G	G	N	V	P	N	V	V	L	E	250
	C	T	G	A	E	P	C	I	K	L	260

5	G	V	D	A	I	A	P	G	G	R	²⁷⁰
10	F	V	Q	V	G	N	A	A	G	P	²⁸⁰
15	V	S	F	P	I	T	V	F	A	M	²⁹⁰
20	K	E	L	T	L	F	G	S	F	R	³⁰⁰
25	Y	G	F	N	D	Y	K	T	A	V	³¹⁰
30	G	I	F	D	T	N	Y	Q	N	G	³²⁰
35	R	E	N	A	P	I	D	F	E	Q	³³⁰
40	L	I	T	H	R	Y	K	F	K	D	³⁴⁰
45	A	I	E	A	Y	D	L	V	R	A	³⁵⁰
	G	K	G	A	V	K	C	L	I	D	³⁶⁰
50	G	P	E	*							

7. The DNA sequence according to claim 6, comprising the following nucleotide sequence:

-310 -290
TCTAGACCACCCCTAAGTCGTCCCTATGTCG

5 -270
TATGTTGCCTCTACTACAAAGTTACTAGC

10 -250 -230
AAATATCCGCAGCAACAAACAGCTGCCCTCT

15 -210
TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

20 -190 -170
CGCTTTCGGGCTCCAGCTTCTGTCCCTCTGC

25 -150
GGCTGCTGCACATAACGCGGGGACAATGAC

30 -130 -110
TTCTCCAGCTTTATTATAAAAGGAGCCAT

35 -90
CTCCTCCAGGTGAAAAATTACGATCAAACCT

40 -70 -50
TTACTCTTCCATTGTCTTGTGTATAC

45 -30
TCACTTTAGTTGTTCAATCACCCCTAAT

50 -10 10
ACTCTTCACACAATTAAAATGACTGCTAAC

55 30
CCTTCCTTGGTGTGAACAAGATCGACGAC

50 70
ATTTCGTTCGAAACTTACGATGCCCGAGAA

90 ATCTCTGAAACCTACCGATGTCCTCGTCCAG

5 110 130
GTCAAGAAAAACCGGTATCTGTGGTTCCGAC

10 150
ATCCACTTCTACGCCCATGGTAGAATCGGT

15 170 190
AACTTCGTTTGACCAAGCCAATGGTCTTG

210
GGTCACGAATCCGCCGGTACTGTTGTCCAG

230 250
GTTGGTAAGGGTGTACCTCTCTTAAGGTT

270

290 310
CCATCCAGATTCTCCGACGAATAACAAGAGC

330

40 350 370
TTCGCCGCTACTCCTAACTCCAAGGAAGGC

45 390
GAACCAAACCCACCAGGTACCTTATGTAAG

50 410 430
TAC TTCAAGTCGCCAGAAGACTTCTGGTC

450
AAGTTGCCAGACCACGTCAGCTTGGAACTC

470 490
GGTGCTCTGTTGAGCCATTGTCTGTTGGT

5 510
GTCCACGCCTCCAAGTTGGGTTCCGTTGCT

10 530 550
TTCGGCGACTACGTTGCCGTCTTGGTGCT

15 570
GGTCCTGTTGGTCTTGGCTGCTGCTGTC

20 590 610
GCCAAGACCTTCGGTGCTAAGGGTGTCA

25 630
GTCGTTGACATTTGACAACAAGTTGAAG

30 650 670
ATGGCCAAGGACATTGGTGCTGCTACTCAC

35 690
ACCTTCAACTCCAAGAACCGGTGGTCTGAA

40 710 730
GAATTGATCAAGGCTTCGGTGGTAACGTG

45 750
CCAAACGTCGTTGGAATGTACTGGTGCT

50 770 790
GAACCTTGTATCAAGTTGGGTGTTGACGCC

55 810
ATTGCCCCCAGGTGGTCGTTCGTTCAAGTT

55 830 850
GGTAACGCTGCTGGTCCAGTCAGCTTCCCA

870
ATCACCGTTTGC~~C~~CATGAAGGAATTGACT

5
890 910
TTGTTCGGTTCTTCAGATA~~C~~GGATTCAAC

10 930
GA~~T~~ACAAGA~~G~~ACTGCTGTT~~G~~GAATCTTGAC

15 950 970
ACTAA~~A~~CTACCAAAACGGTAGAGAAAATGCT

20 990
CCAATTGACTTTGAACAA~~T~~GATCACCCAC

25 1010 1030
AGATACAA~~G~~TTCAAGGACGCTATTGAAGCC

30 1050
TACGACTTGGTCAGAGCCGGTAAGGGTGCT

35 1070 1090
GTCAAGTGTCTCATTGACGGCCCTGAGTAA

40 1110
GTCAACC~~G~~CTTGGCTGGCCAAAGTGAACC

45 1130 1150
AGAA~~A~~CGAAAATGATTATCAAATAGCTTTA

50 1170
TAGACCTTATCGAAATTATGTAA~~A~~CTAA

55 1190 1210
TAGAAAAGACAGTGTAGAAGTTATATGGTT

1230
55 GCATCACGTGAGTTCTGAATTCTGAAA

5 1250 1270
GTGAAGTCTTGGTCGGAACAAACAAACAAA

10 1290
AAAATATTTCAGCAAGAGTTGATTCTTT

15 1310 1330
TCTGGAGATTTGGTAATTGACAGAGAACCC

20 1350
CCTTTCTGCTATTGCCATCTAACACATCCTT

25 1370 1390
GAATAGAACCTTACTGGATGGCCGCCTAGT

30 1410
GTTGAGTATATATTATCAACCAAAATCCTG

35 1430 1450
TATATAGTCTCTGAAAAATTGACTATCCT

40 1470
AACTTAACAAAAGAGCACCCATAATGCAAGC

45 1490 1510
TCATAGTTCTTAGAGACACCAACTATACTT

50 1530
AGCCAAACAAAATGTCCTTGGCCTCTAAAG

55 1550 1570
AAGCATTCAAGCAAGCTTCCCCAGAAGTTGC

60 1590
ACAACCTCTTCATCAAGTTACCCCCAGAC

65 1610 1630
CGTTTGCCGAATATTGGAAAAGCCTTCGA

70 CTATAGTGGATCC

8. The DNA sequence according to any of claims 1 to 7, characterized in that it is obtained by recombinant DNA technology from natural and/or cDNA and/or chemically synthesised DNA.
- 5 9. A combination of DNA sequences, characterized in that said combination comprises a first DNA sequence according to any of claims 1 to 8 and one or more further DNA sequences capable of regulating the expression of a structural gene encoded by said DNA sequence in a host microorganism.
- 10 10. A combination of DNA sequences according to claim 9, characterized in that said combination comprises all modifications of the DNA sequences retaining their capability to express a functional enzyme having xylose reductase or xylitol dehydrogenase activity.
- 15 11. A combination of DNA sequences according to claim 9 or 10, characterized in that said structural gene contains DNA sequences derived from the structural gene coding for xylose reductase or xylitol dehydrogenase which modify said protein product while retaining its functions in such a way that said protein product is expressed as a gene product having enzymatic activity.
- 20 12. A combination of DNA sequences according to any of claims 8 to 11, characterized in that said DNA sequences capable of regulating the expression of said structural gene in a host microorganism are derived from said host microorganism.
13. A combination according to claim 12, characterized in that said DNA sequences capable of regulating the expression are inducible promoters.
- 25 14. A combination according to claims 13, characterized in that said DNA sequences capable of regulating the expression are selected from the following promoters: ADH1, ADH2, PDC, GAL1/10.
15. A combination according to any of claims 12 to 14 characterized in that said DNA sequence capable of regulating the expression of said structural gene is a strong promoter, leading to over expression of the protein encoded by said structural gene.
- 30 16. A vector, characterized in that said vector comprises DNA sequences according to any of claims 1 to 8 or a combination of DNA sequences according to any of claims 9 to 15.
- 35 17. A vector according to claim 16, characterized in that said vector is selected from the group comprising the plasmids pR1, pR2, pD1, pD2, pRD1, pXR_a, pXR_b pXD_H, pXR, pXD_H-HIS3, pXR-LEU2.
18. A microorganism, characterized in that said microorganism is capable of expressing a xylose reductase and/or a xylitol dehydrogenase as a result of having received DNA sequences comprising the DNA sequences according to any of claims 1 to 8 or a combination of DNA sequences according to any of claims 9 to 15, coding for said xylose reductase and/or xylitol dehydrogenase by recombinant DNA technology.
- 40 45 19. The microorganism according to claim 18, characterized in that said microorganism is selected from a group consisting of yeast of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen or Paecilomyces or bacteria of the genus Zymomonas.
- 50 20. The microorganism according to claim 19, characterized in that said microorganism is Saccharomyces cerevisiae.
21. The microorganism according to claim 19, characterized in that said microorganism is Schizosaccharomyces pombe.
- 55 22. The microorganism according to any of claims 18 to 21, characterized in that said DNA sequence or combination of DNA sequences is integrated into the genome of said microorganism.

23. The microorganism according to any of claims 18 to 22, characterized in that said microorganism is useful in biomass production, in food industry and fermentation processes.
- 5 24. The microorganism according to claim 23, characterized in that said microorganism is useful for fermentation of xylose into ethanol.
- 10 25. A method for producing xylose reductase and/or xylitol dehydrogenase by cultivating a microorganism according to any of claims 18 to 22 under suitable conditions and recovering said enzyme(s) in a manner known per se.
- 15 26. The method according to claim 25, characterized in that said microorganism is selected for efficient fermentation of xylulose.
- 20 27. The method according to claim 25 or 26, characterized in that said microorganism has received said DNA sequences or said combination of DNA sequences by transformation using a vector, said vector being preferably a DNA fragment or a plasmid.
28. The method according to claim 27, characterized in that said vector contains DNA, which is homologous to DNA of said microorganism, leading to integration into the genome of said microorganism.
29. A xylose reductase produced according to the method of any of claims 25 to 28.
30. A xylitol dehydrogenase produced according to the method of any of claims 25 to 28.
- 25 31. An ethanol manufacturing process, characterized in that a microorganism according to any of claims 18 to 24 is used.
32. A process according to claim 31, characterized in that the fermentation process is adapted for the production of alcoholic beverages or single cell protein produced from substrates containing free xylose, preferably released by xylanase and/or xylosidase activity.
- 30 33. A process for production of biomass, characterized in that a host microorganism according to any of claims 18 to 24 is used.
- 35 34. A process for recycling of NADP⁺ from NADPH using xylose reductase.
36. A method for producing a desired protein in Pichia stipitis, comprising expression of the structural gene encoding said desired protein under control of the 5' regulating region of the XYL1 and/or XYL2 gene of Pichia stipitis and/or the ADH1 promoter from Saccharomyces cerevisiae and/or the glucoamylase promoter from Schwanniomyces occidentalis.

45

50

55

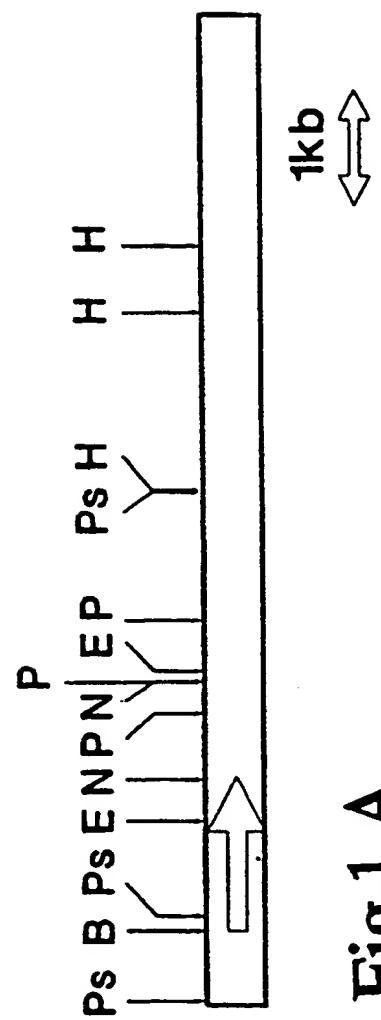


Fig. 1 A

Fig.1 B

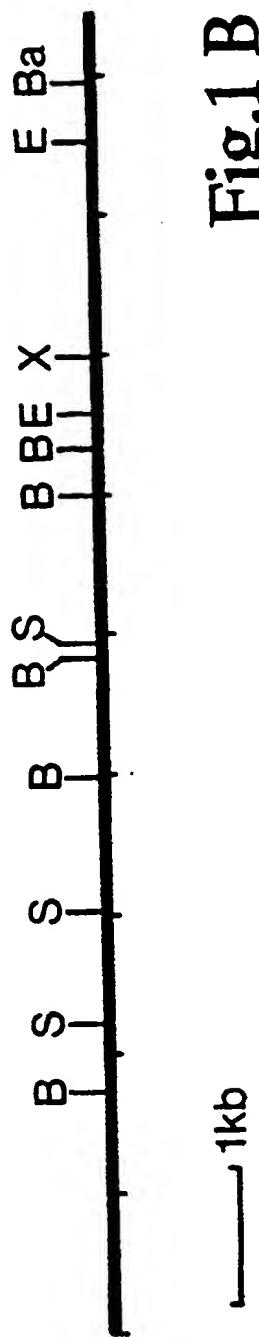


Fig.2A (1)

-350
GGATCCACAGACACTAATTGGTTCTA

-310
CATTATTCGTGTTCAGACACAAACCCCAGC

-290
GTTGGCGGTTCTGTCTGCCTCCAGC

-250
ACCTTCTTGCTCAACCCCCAGAAGGTGCACA

-230
CTGCAGACACACATAACATAACGAGAACCTGG

-190
AACAAATATCGGTGTCGGTGACCGAAATGT

-170
GCAAACCCAGACACGACTAATAAACCTGGC

-130
AGCTCCAATACCGCCGACAAACAGGTGAGGT

-110
GACCGATGGGGTGCCAATTAATGTCTGAAA

-70
ATTGGGGTATATAAATATGGCGATTCTCCG

-50
GAGAATTTTCAGTTTCTTTCATTTCTC

-10
CAGTATTCTTTCTATACAACCTACTACA

10	30
ATGCCTTCTATTAAGTTGA	ACTCTGGTTAC
M P S I K L N S G Y	

Fig.2A (2)

50

GACATGCCAGCCGTCGGTTTCGGCTGTTGG
D M P A V G F G C W

70 90

AAAGTCGACGTCGACACCTGTTCTGAACAG
K V D V D T C S E Q

110

ATCTACCGTGCTATCAAGACCGGTTACAGA
I Y R A I K T G Y R

130 150

TTGTTCGACGGTGCCGAAGATTACGCCAAC
L F D G A E D Y A N

170

GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG
E K L V G A G V K K

190 210

GCCATTGACGAAGGTATCGTCAAGCGTGAA
A I D E G I V K R E

230

GACTTGTTCCTTACCTCCAAGTTGTGGAAC
D L F L T S K L W N

250 270

AACTACCACCAACCCAGACAACGTCGAAAAG
N Y H H P D N V E K

290

GCCTTGAACAGAACCCCTTCTGACTTGCAA
A L N R T L S D L Q

310 330

GTTGACTACGTTGACTTGTCTGATCCAC
V D Y V D L F L I H

Fig.2A (3)

350

TTCCCAGTCACCTCAAGTTCGTCCATT
F P V T F K F V P L

370 390

GAAGAAAAGTACCCACCAGGATTCTACTGT
E E K Y P P G F Y C

410

GGTAAGGGTGACAACCTCGACTACGAAGAT
G K G D N F D Y E D

430 450

GTTCCAATTTAGAGACCTGGAAGGCTCTT
V P I L E T W K A L

470

GAAAAGTTGGTCAAGGCCGGTAAAGATCAGA
E K L V K A G K I R

490 510

TCTATCGGTGTTCTAACCTCCCAGGTGCT
S I G V S N F P G A

530

TTGCTCTGGACTTGTGAGAGGTGCTACC
L L L D L L R G A T

550 570

ATCAAGCCATCTGTCTGCAAGTTGAACAC
I K P S V L Q V E H

590

CACCCATACTTGCAACAAACCAAGATTGATC
H P Y L Q Q P R L I

610 630

GAATTCGCTCAATCCCGTGGTATTGCTGTC
E F A Q S R G I A V

Fig.2A (4)

650
 ACCGCTTACTCTCGTCGGCCTCAATCT
 T A Y S S F G P Q S

670 690
 TTCGTTGAATTGAACCAAGGTAGAGCTTG
 F V E L N Q G R A L

710
 AACACTTCTCCATTGTTCGAGAACGAAACT
 N T S P L F E N E T

730 750
 ATCAAGGCTATCGCTGCTAACGCACGGTAAG
 I K A I A A K H G K

770
 TCTCCAGCTCAAGTCTTGTGAGATGGTCT
 S P A Q V L L R W S

790 810
 TCCCAAAGAGGCATTGCCATCATTCCAAAG
 S Q R G I A I I P K

830
 TCCAACACTGTCCCAGATTGTTGGAAAAC
 S N T V P R L L E N

850 870
 AAGGACGTCAACAGCTCGACTTGGACGAA
 K D V N S F D L D E

890
 CAAGATTCGCTGACATTGCCAAGTTGGAC
 Q D F A D I A K L D

910 930
 ATCAAACATTGAGATTCAACGACCCATGGGAC
 I N L R F N D P W D

Fig.2A (5)

950
TGGGACAAGATTCCCTATCTTCGTCTAAGAA
 W D K I P I F V *

970 990
GGTTGCTTATAGAGAGGAAATAAACCTA

1010
ATATACATTGATTGTACATTTAAAATTGAA

1030 1050
TATTGTAGCTAGCAGATTCGGAAATTAAA

1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

1090 1110
TCTCTATGTACATACACGTTGAAGATAGCA

1130
GTACAGTAGACATCAAGTCTACAGATCATT

1150 1170
AAACATATCTTAAATTGTAGAAAACTATAA

1190
ACTTTCAATTCAAACCATGTCTGCCAAGG

1210 1230
AATCAAATGAGATTTTCGCAGCCAAAC

1250
TTGAATCCAAAAATAAAAAACGTCTTGTC

1270 1290
TGAAACAACTCTATCTTACACCTCA

1310
TCAATTGATATCATAAAAGCCTCCG

Fig.2A (6)

1330 1350
 ATAGCATAACAAACTACTTCTGCATCATAT

1370
 CTAAATCATAGTGCCATATTCAAGAACAAAT

1390 1410
 ACCGGTAAGAAACTTCTATTTTTTAGTCT

1430
 GCCTTAACGAGATGCAGATCGATGCAACGT

1450 1470
 AAGATCAAACCCCTCCAGTTGTACAGTCAG

1490
 TCATATAGTGAACACCGTACAATATGGTAT

1510 1530
 CTACGTTCAAATAGACTCCAATACAGCTGG

1550
 TCTGCCAAGTTGAGCAACTTTAATTAGA

1570 1590
 GACAAAGTCGTCTCTGTTGATGTAGGCACC

1610
 ACACATTCTTCTTTGCCCGTGAACCTGT

1630 1650
 TCTGGAGTGGAAACATCTCCAGTTGTAAA

1670
 TATCAAACACTGACCAGGCTCAACTGGTA

1690
 GAAGATTCGTTTGGGATCC

Fig.2B (1)

-310 -290

TCTAGACCACCCTAACAGTCGTCCCTATGTCG

-270

TATGTTGCCTCTACTACAAAGTTACTAGC

-250 -230

AAATATCCGCAGCAACAAACAGCTGCCCTCT

-210

TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

-190 -170

CGCTTTGGGCTCCAGCTTCTGTCCTCTGC

-150

GGCTGCTGCACATAACGCGGGGACAATGAC

-130 -110

TTCTCCAGCTTTATTATAAAAAGGAGCCAT

-90

CTCCTCCAGGTGAAAAATTACGATCAACTT

-70 -50

TTACTCTTCCATTGTCTCTGTGTATAC

-30

TCACTTTAGTTGTTCAATCACCCCTAAT

-10 10

ACTCTTCACACAATTAAAATGACTGCTAAC
M T A N

30

CCTTCCTTGGTGTGAACAAAGATCGACGAC
P S L V L N K I D D

Fig.2B (2)

50 70

ATTCGTTCGAAACTTACGATGCCAGAA
I S F E T Y D A P E

90

ATCTCTGAACCTACCGATGTCCCTCGTCCAG
I S E P T D V L V Q

110 130

GTCAAGAAAACCGGTATCTGTGGTCCGAC
V K K T G I C G S D

150

ATCCACTTCTACGCCATGGTAGAATCGGT
I H F Y A H G R I G

170 190

AACTTCGTTTGACCAAGCCAATGGTCTTG
N F V L T K P M V L

210

GGTCACGAATCCGCCGGTACTGTTGTCCAG
G H E S A G T V V Q

230 250

GTTGGTAAGGGTGTACCTCTCTTAAGGTT
V G K G V T S L K V

270

GGTGACAACGTCGCTATCGAACCAAGGTATT
G D N V A I E P G I

290 310

CCATCCAGATTCTCCGACGAATAACAAGAGC
P S R F S D E Y K S

330

GGTCACTACAACCTGTGTCCACATGGCC
G H Y N L C P H M A

Fig.2B (3)

350 370

TTCGCCGCTACTCCTAACTCCAAGGAAGGC
F A A T P N S K E G

390

GAACCAAACCCACCAGGTACCTTATGTAAG
E P N P P G T L C K

410 430

TACTTCAAGTCGCCAGAAGACTTCTGGTC
Y F K S P E D F L V

450

AAGTTGCCAGACCACGTCAGCTTGGAACTC
K L P D H V S L E L

470 490

GGTGCTCTTGTGAGCCATTGTCTGTTGGT
G A L V E P L S V G

510

GTCCACGCCTCCAAGTTGGGTTCCGTTGCT
V H A S K L G S V A

530 550

TTCGGCGACTACGTTGCCGTCTTGGTGCT
F G D Y V A V F G A

570

GGTCCTGTTGGTCTTTGGCTGCTGCTGTC
G P V G L L A A A V

590 610

GCCAAGACCTTCGGTGCTAAGGGTGTCAATC
A K T F G A K G V I

630

GTCGTTGACATTTGACAACAAGTTGAAG
V V D I F D N K L K

Fig.2B (4)

650 670
 ATGGCCAAGGACATTGGTGCTGCTACTCAC
 M A K D I G A A T H

690
 ACCTTCAACTCCAAGAACCGGTGGTCTGAA
 T F N S K T G G S E

710 730
 GAATTGATCAAGGCTTCGGTGGTAACGTG
 E L I K A F G G N V

750
 CCAAACGTCGTTTGGAAATGTACTGGTGCT
 P N V V L E C T G A

770 790
 AACCTTGTATCAAGTTGGGTGTTGACGCC
 E P C I K L G V D A

810
 ATTGCCCCAGGTGGTCGTTCAAGTT
 I A P G G R F V Q V

830 850
 GGTAAACGCTGCTGGTCCAGTCAGCTTCCCA
 G N A A G P V S F P

870
 ATCACCGTTTCGCCATGAAGGAATTGACT
 I T V F A M K E L T

890 910
 TTGTTGGTTCTTCAGATAACGGATTCAAC
 L F G S F R Y G F N

930
 GACTACAAGACTGCTGTTGGAATCTTGAC
 D Y K T A V G I F D

Fig.2B (5)

950 970
ACTAACTACCAAAACGGTAGAGAAAATGCT
 T N Y Q N G R E N A

990
CCAATTGACTTTGAACAATTGATCACCCAC
 P I D F E Q L I T H

1010 1030
AGATACAAAGTTCAAGGACGCTATTGAAGCC
 R Y K F K D A I E A

1050
TACGACTTGGTCAGAGCCGGTAAGGGTGCT
 Y D L V R A G K G A

1070 1090
GTCAAAGTGTCTCATTGACGGCCCTGAGTAA
 V K C L I D G P E *

1110
GTCAACCGCTTGGCTGGCCAAAGTGAACC

1130 1150
AGAAACGAAAATGATTATCAAATAGCTTTA

1170
TAGACCTTATCGAAATTATGTAACTAA

1190 1210
TAGAAAAGACAGTGTAGAAGTTATGGTT

1230
GCATCACGTGAGTTCTTGAATTCTTGGAAA

1250 1270
GTGAAGTCTTGGTCGGAACAAACAAACAAA

1290
AAAATATTTTCAGCAAGAGTTGATTTCTT

Fig.2B (6)

1310 1330
TCTGGAGATTTGGTAATTGACAGAGAAC

1350
CCTTTCTGCTATTGCCATCTAAACATCCTT

1370 1390
GAATAGAACTTTACTGGATGGCCGCCTAGT

1410
GTTGAGTATATATTATCAACCAAAATCCTG

1430 1450
TATATAGTCTCTGAAAAATTGACTATCCT

1470
AACTTAACAAAAGAGCACCATATAATGCAAGC

1490 1510
TCATAGTTCTTAGAGACACCAACTATACTT

1530
AGCCAAACAAAATGTCCTGGCCTCTAAAG

1550 1570
AAGCATTTCAGCAAGCTTCCCCAGAAGTTGC

1590
ACAACTTCTTCATCAAGTTACCCCCAGAC

1610 1630
CGTTTGCCGAATATTGGAAAAGCCTTCGA

CTATAGTGGATCC

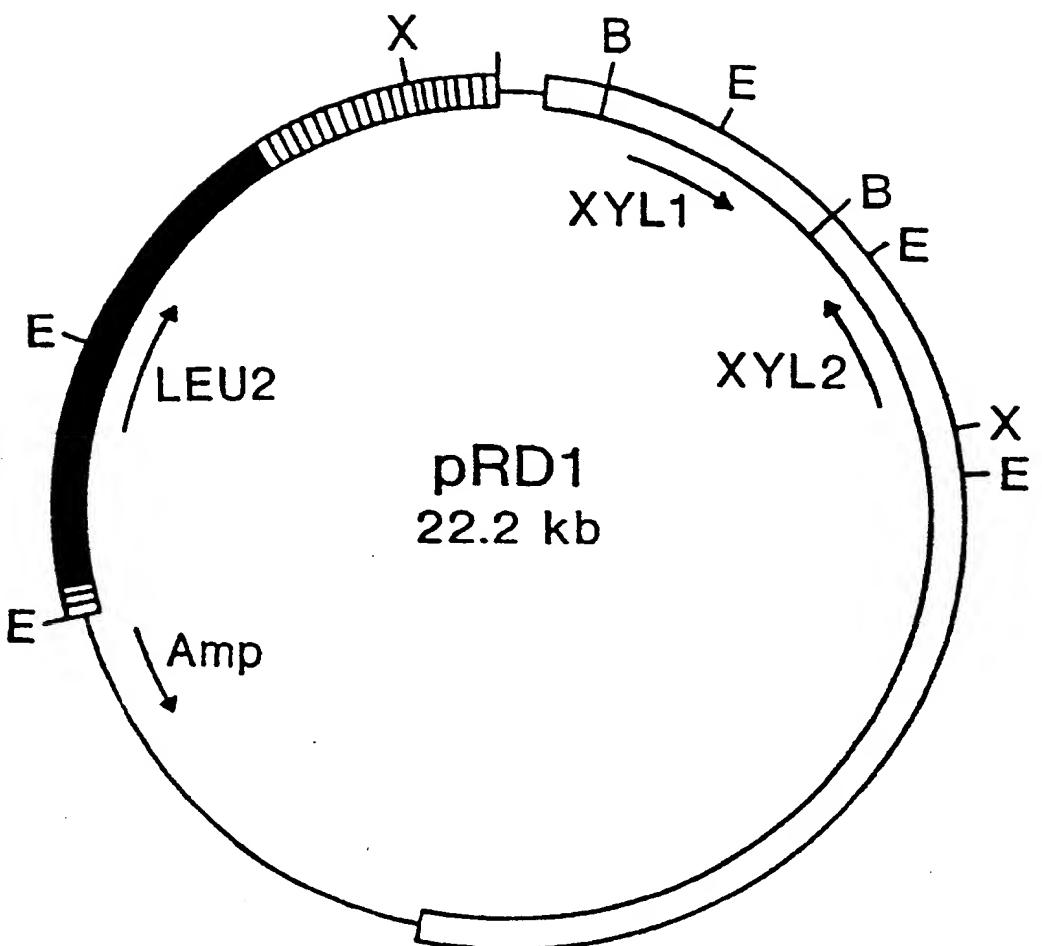


Fig.3

- [White box] *P. stipitis*
- [Black box] *S. cerevisiae*
- [Hatched box] 2 μ
- pBR322

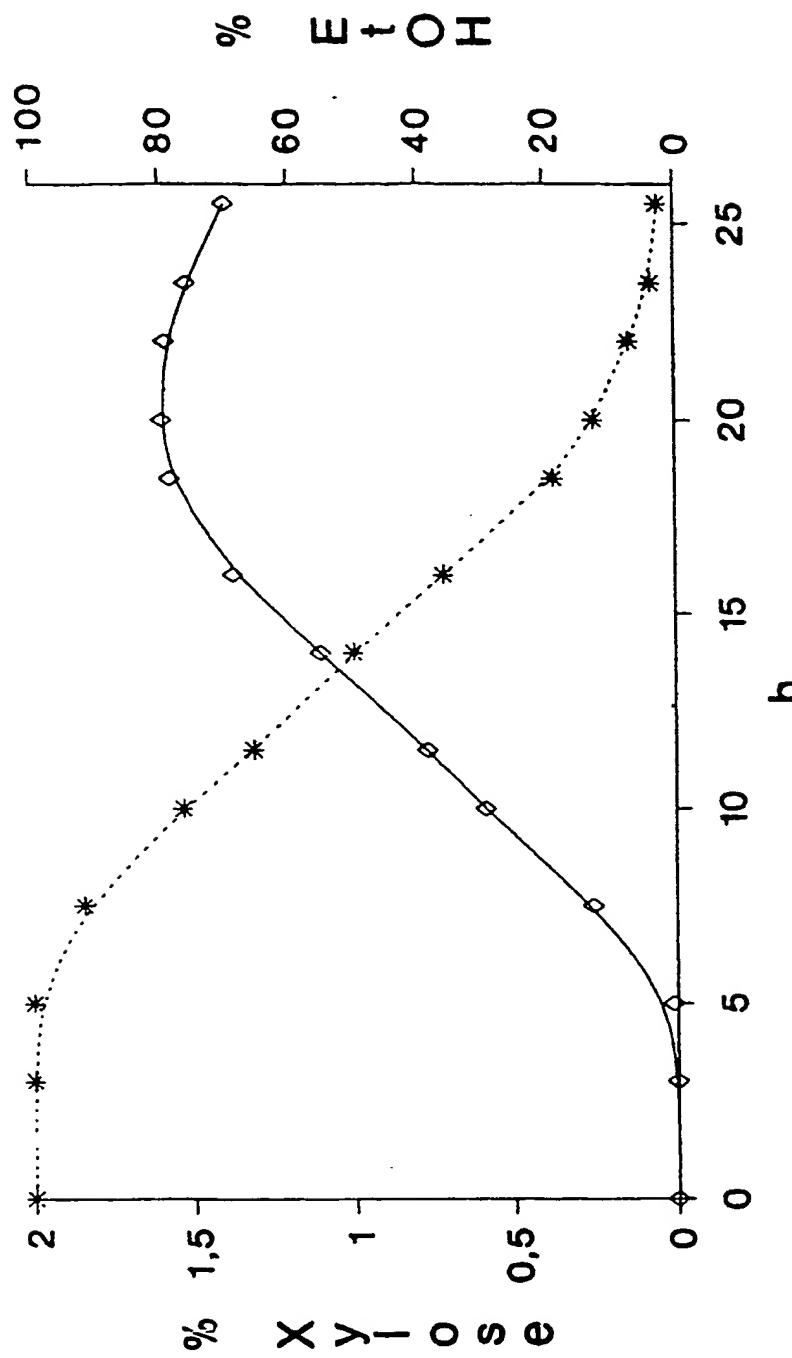


Fig.4 ...*... Xylose ——♦— Ethanol

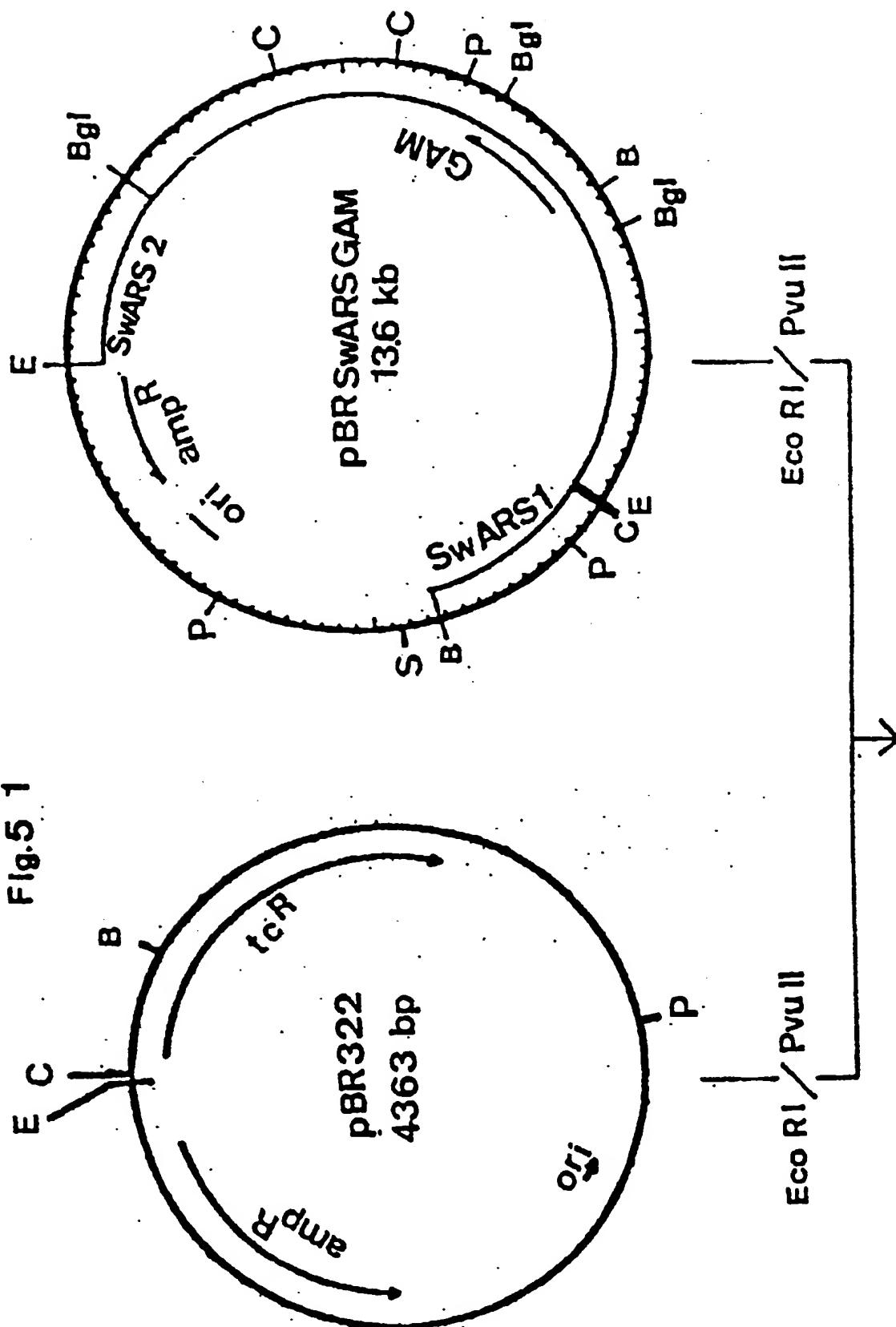
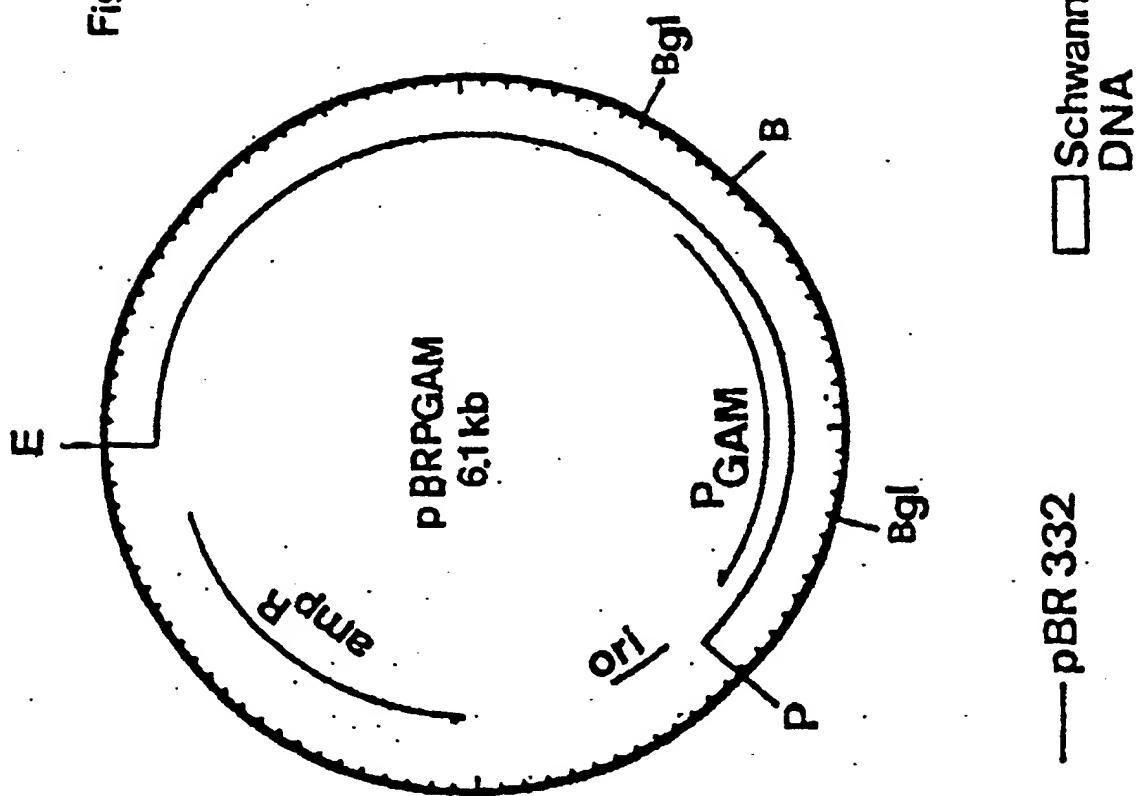


Fig.6 2



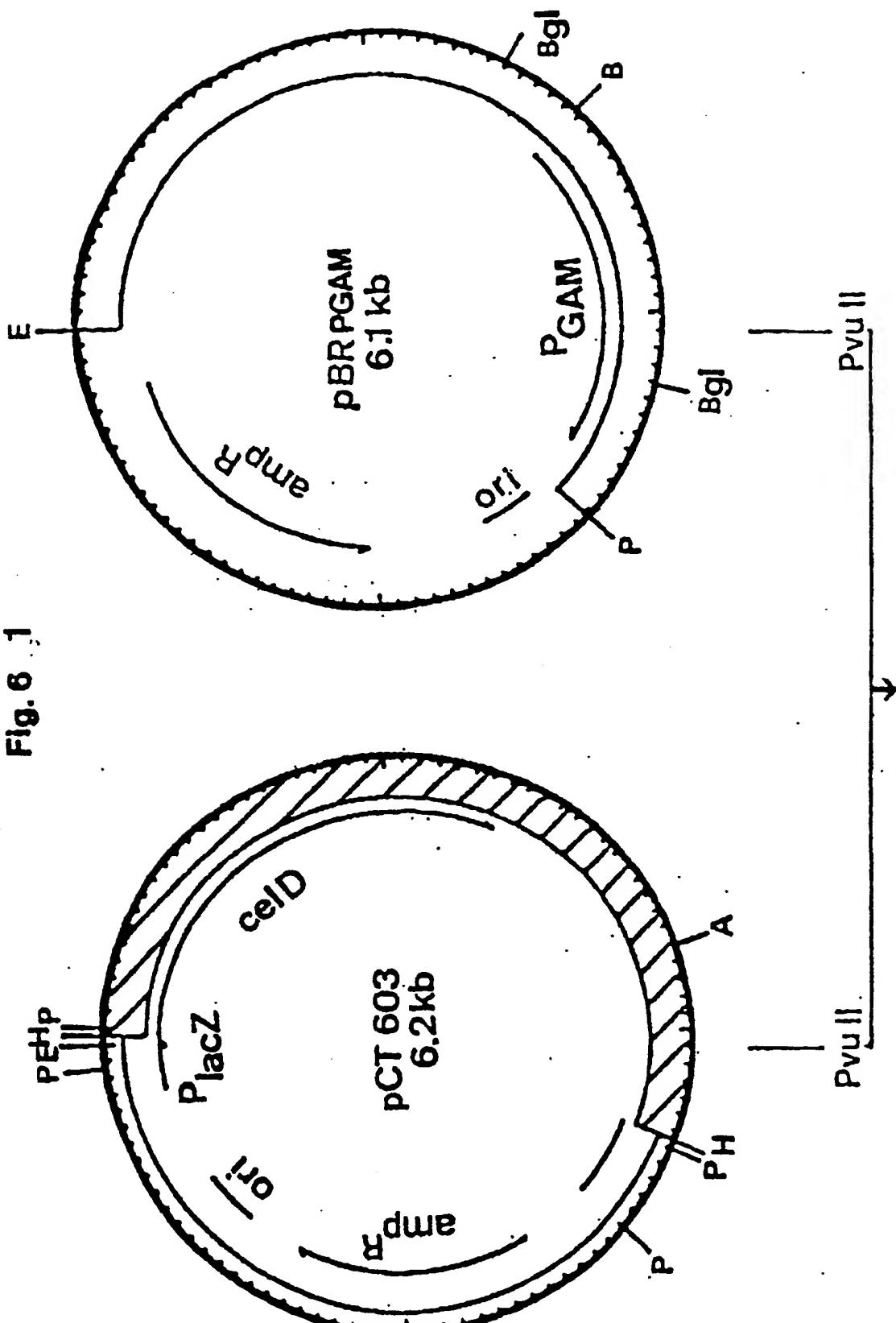
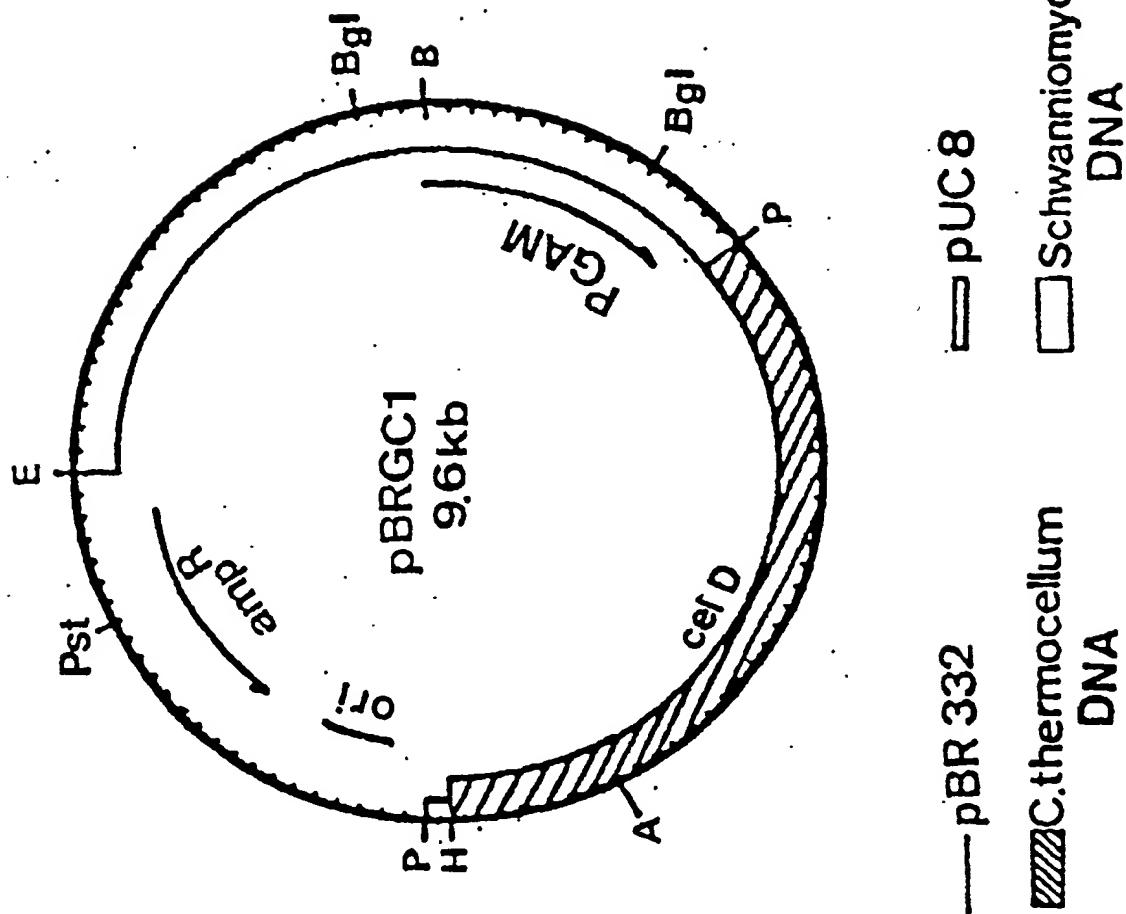


Fig 6 ,2



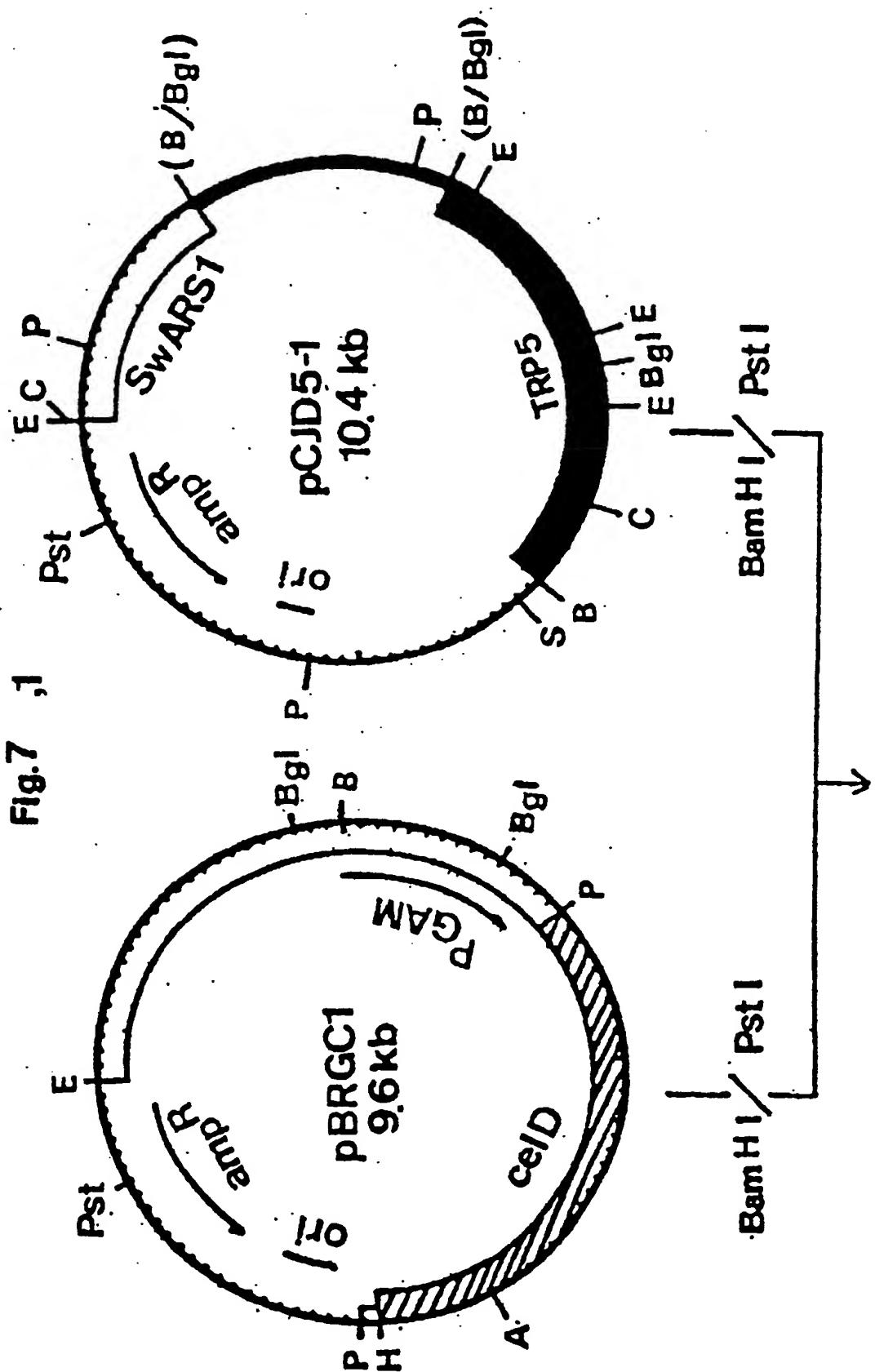
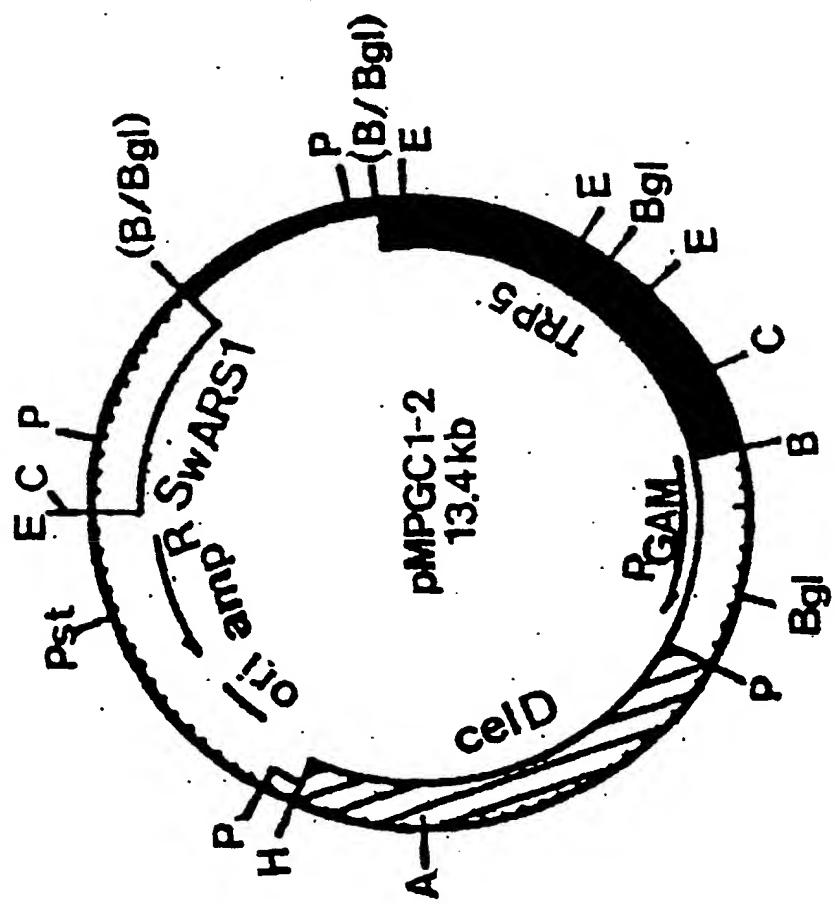
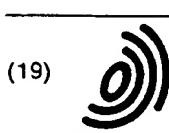


Fig. 7., 2



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(54) DNA sequence comprising a structural gene coding for xylose reductase or xylose reductase and xylitol dehydrogenase

DNS Sequenz, bestehend aus einem kodierenden, strukturellen Gen für Xylose-Reduktase oder Xylose -Reduktase und Xylitol-Dehydrogenase

Séquence d'ADN comprenant un gène codant pour la réductase de xylose ou la réductase de xylose et la déhydrogénase de xylitol

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Description

The present invention relates to a DNA sequence, a combination of DNA sequences, a vector, a microorganism, a method for producing xylose reductase or xylose reductase and xylitol dehydrogenase; the invention further relates to an ethanol manufacturing process, a process for production of biomass, a process for recycling of NADP⁻ from NADPH and a method for producing a desired protein in *Pichia stipitis*.

D-xylose is one of the most abundant carbohydrates occurring in plant biomass and wood. In the process of cel-lulose production, it is formed as a waste product from hydrolysis of xylan, which is the major compound of hemicel-lulose. To optimize the use of renewable carbon sources, it is desirable to convert xylose into ethanol or biomass.

There are several yeast species, such as *Candida* (Gong et al., 1981, Jeffries, 1983), *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pachysolen*, *Paecilomyces* (Wu et al., 1986) and *Pichia* (Maleszka and Schneider 1982), which are able to utilize pentoses, including D-xylose, and D-ribose, however, only aerobically.

In general, pentoses utilized by yeasts (e.g. *Pichia stipitis*) must be isomerized to pentuloses in order to be phosphorylated. This isomerization occurs via a NAD(P)H linked reduction (reductase) to pentitols followed by NAD⁺-linked oxidation (dehydrogenase) of the pentitols to the corresponding D-pentuloses (Barnett, 1976). The yeast mainly used in bioethanol production, *S. cerevisiae*, can utilize xylulose, however, this yeast is not able to ferment pentoses (Jeffries, 1988). It cannot be excluded, that *S. cerevisiae* also contains genes, coding for pentose fermenting proteins which however are not expressed.

Pentose fermentation by *S. cerevisiae* may be possible by providing a xylose utilising pathway from a xylose metabolizing organism. However, although many attempts have been undertaken to express bacterial xylose isomerase genes in *S. cerevisiae*, no xylose fermentation could be obtained probably due to inefficient expression of the foreign gene (Sarthy et al., 1987, Amore et al., 1989, Chan et al., 1986 & 1989).

Therefore it is a primary object of the present invention to provide genes of the enzymes involved in xylose degradation in order to be able to manipulate these genes, for example to combine these sequences with suitable regulating sequences.

This object has been solved by a DNA sequence comprising a structural gene coding for xylose reductase or xylose reductase and xylitol dehydrogenase and being capable of expressing said polypeptide(s) in a microorganism.

Further objects of the present invention will become apparent by the following detailed description of the invention, the examples and figures.

Throughout this application various publications are referenced by the first author within parenthesis.

Full citations of these references may be found at the end of the specification as an annex. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The DNA sequences according to the present invention preferably are derived from a yeast. Preferred yeast strains are selected from the genera *Schwanniomyces*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Hansenula*, *Candida*, *Debaryomyces*, *Metschnikowia*, *Pachysolen* and *Paecilomyces*. All of these yeast genera are known to be able to convert xylose into ethanol using xylose reductase and xylitol dehydrogenase.

A preferred genus used as a source for the DNA sequence according to the present invention is the yeast *Pichia*. This genus comprises several species, any of which could be applied for performing the present invention. However, the preferred species is *Pichia stipitis*. The present inventors used *Pichia stipitis* CBS5773 for isolation of the DNA sequences comprising a structural gene coding for xylose reductase and/or xylitol dehydrogenase. *Pichia stipitis* CBS5773 was redeposited under the Budapest Treaty on March 21, 1990 (DSM 5855).

The present inventors succeeded to isolate DNA molecules containing a sequence comprising the structural gene encoding a xylose reductase and a xylitol dehydrogenase respectively. By way of the DNA sequence, which was determined according to standard procedures, the amino acid sequence of both these proteins could be determined for the first time. The complete amino acid sequences as well as the nucleotides sequences of both these proteins are shown in Figures 2A and 2B. As is known to everybody skilled in the art the proteins having the amino acid sequences as shown in Figures 2A and 2B can be encoded not only by the DNA sequences as found in *Pichia stipitis* CBS5773, but also by using alternative codons provided by the degeneracy of the genetic code. The invention thus is not limited to the DNA sequence as shown in Figure 2, but also comprises any modification yielding the same amino acid sequences.

The DNA sequences according to the present invention may not only be obtained by applying the methods shown below, i.e., by isolating cDNA clones, which further on are used to screen a genomic library, but also may be obtained by other methods of recombinant DNA technology from either natural DNA or cDNA or chemically synthesized DNA or by a combination of two or more of these DNAs. For example, it may be attempted to combine a chemically synthesized 5' region with a cDNA coding for the 3' region or any other combination of the three DNA sources mentioned above.

According to the present invention there are also provided combinations of DNA sequences, which comprise a

DNA sequence as discussed above, i.e., a sequence comprising a structural gene coding for a xylose reductase or xylose reductase and xylitol dehydrogenase, and in addition one or more DNA sequences capable of regulating the expression of the structural genes mentioned above in a presumptive host microorganism. DNA sequences capable of regulating the expression of structural genes are well known to those skilled in the art. For example, the DNA sequences discussed above may be combined with promoters, which are connected with the structural genes in order to provide efficient expression. Further DNA sequences capable of regulating the expression may comprise enhancers, termination sequences and polyadenylation signals. Examples for the best known kind of regulating sequences, are shown by the following examples.

In order to express the DNA sequences and/or the combination of DNA sequences according to the present invention efficiently, small modifications of the DNA sequences may be performed, as long as their capability to express a functional enzyme having the desired xylose reductase or xylitol dehydrogenase activity is retained. These modifications may include either variations of the genetic code as discussed above or furthermore small substitutions of the amino acid sequence, as well as deletions and/or insertions, which do not have any detrimental impact on the respective enzyme activity.

In a preferred embodiment the DNA sequence, capable of regulating the expression of the structural gene, is derived from an endogenous gene of the microorganism, in which expression of the DNA sequence is intended. Since, as will be shown below in more detail, Saccharomyces cerevisiae is one of the preferred microorganism to be used in the present invention, there are a multitude of possible regulating sequences known. Some of these well-known sequences have been used to construct expression vectors, as will be shown below in the examples. In the most preferred embodiment the combination of DNA sequences comprises inducible promoters. In this case the expression of xylose reductase and xylitol dehydrogenase can be prevented, as long as desired; expression may be started upon addition of a suitable inducer.

In the most preferred embodiments of the present invention the following Saccharomyces cerevisiae promoters are used to regulate the expression of the genes encoding xylose reductase or xylose reductase and xylitol dehydrogenase: ADH1, ADH2, PDC, GAL1/10.

Depending on the choice of the respective promoter it may be possible to obtain expression levels exceeding that of natural expression of both proteins in their original host organism.

The DNA sequences as well as the combinations of the DNA sequences according to the present invention may be introduced in vector molecules. These molecules may be plasmids, which are suitable for replication in the desired host microorganism and thus should contain a functional origin of replication. Alternatively, it is also possible, to use linear DNA fragments carrying the DNA sequence or combination of DNA sequences according to the present invention or to use circular DNA molecules being devoid of a functional origin of replication. In this case the vector, which is not capable of replication, will be inserted by either homologous or nonhomologous recombination into the host chromosome.

Subject of the present invention are further microorganisms, which have received DNA sequences comprising the inventive DNA sequences or combinations of DNA sequences coding for xylose reductase and xylitol dehydrogenase by recombinant DNA technology.

Preferred microorganisms are selected from a group consisting of yeast of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen or Paecilomyces or bacteria of the genus Zymomonas.

From these organisms the most preferred microorganisms are Saccharomyces cerevisiae and Schizosaccharomyces pombe and Zymomonas.

One of the possible applications of the genetically altered yeast strains described above is the production of biomass. Since the yeast strains having acquired the ability of expressing xylose reductase or xylose reductase and xylitol dehydrogenase are maintaining good fermentation abilities, biomass can be produced most efficiently by use of these inventive yeast strains. The methods for producing biomass are the usual ones, which are known to everybody skilled in the art. The genetically manipulated yeast strains provided in compliance with this invention are also suitable for the production of ethanol. The preferred organisms for use in the production of ethanol by fermentation are the yeasts Saccharomyces cerevisiae and/or Schizosaccharomyces pombe and/or the bacterium Zymomonas.

The preferred carbohydrate in the ethanol production is xylose. Thus, strains of Saccharomyces cerevisiae and/or Schizosaccharomyces pombe and/or Zymomonas being able to ferment xylose are highly advantageous in the production of ethanol. The production of potable spirit or industrial ethanol by use of a genetically manipulated yeast strain according to the present invention can be carried out in a manner known per se. The inventive yeast strains have the ability to ferment concentrated carbohydrate solutions, exhibit high ethanol tolerance and have the ability of producing elevated concentrations of ethanol; they have a high cell viability for repeated recycling and exhibit remarkable pH-and temperature tolerance. In the process of xylose production xylose is formed as a waste product from hydrolysis of xylan, which is the major compound of hemicellulose. Hence it is of great advantage to use xylose for the production of ethanol and/or biomass. The invention is further suitable for the production and isolation of the NAD(P)

H linked xylose reductase. Due to the reduction reaction this enzyme is suitable for the delivering or recycling (from NADPH to an NADP⁺) of the corresponding coenzyme especially in bioreactors, for example for the production of amino acids.

5 A further subject of the present invention is a method for producing the xylose reductase or xylose reductase and xylitol dehydrogenase by cultivating a microorganism according to the present invention under suitable conditions and recovering said enzyme or both of them in a manner known *per se*. The method thus includes the expression of a DNA sequence or a combination of DNA sequences according to the present invention in a suitable microorganism, cultivating said microorganism under appropriate conditions and isolating the enzyme.

10 It could be shown, that the level of expression of desired proteins in the inventive microorganisms is enhanced, if the microorganism has been selected for efficient fermentation of xylulose. Thus, it is preferred, to perform the method for reproducing one or both of the proteins using microorganisms, which have been selected accordingly.

15 Since the present invention provides the cloned genes and the corresponding sequences, the gene products can be overproduced in other organisms, e.g. in yeasts of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen, Paecilomyces or bacteria of the genus Zymomonas. The techniques employed for obtaining expression of the XYL1 (xylose reductase) or XYL1 and XYL2 (xylitol dehydrogenase) gene and the isolation of the active gene product are the usual ones such as promoter-fusion, transformation, integration and selection, and methods of protein isolation, known by the man skilled in the art.

20 Generally, said microorganisms have received the DNA sequence or combination of DNA sequences via transformation procedures. For each of the possible microorganisms, i.e. the different yeast genera and bacteria of the genus Zymomonas, there are transformation procedures known. The transformation is preferably carried out using a vector, which may be either a linear or circular DNA molecule; in addition, the method can be performed using autonomously replicating or integrative molecules as well. In the case, that the molecule is supposed to integrate into the genome of the respective host, it is preferred, to use a vector containing DNA, which is homologous to the DNA of said intended host microorganism. This measure facilitates homologous recombination.

25 Further subjects of the present invention are the enzymes produced according to the above described method.

The microorganisms according to the present invention may be used in ethanol manufacturing processes. Since xylose is a readily available source, which normally is considered to be waste, the ethanol manufacturing process according to the present invention provides a possibility for ethanol production of high economical and ecological interest.

30 The ethanol manufacturing process may be adapted for the production of alcoholic beverages or single cell protein from substrates containing free xylose, which is preferably released by xylanase and/or xylosidase activity from xylan.

According to the present invention there is further provided a method for the production of a desired protein in Pichia stipitis. According to this method a structural gene coding for a desired protein is expressed under control of the 5' regulating region of the XYL1 and/or XYL2 gene from Pichia stipitis and/or the ADH1 promoter of S. cerevisiae and/or the glucoamylase promoter from Schwanniomyces occidentalis. Out of the promoters mentioned before use of the 5' regulating regions of the XYL1 or XYL2 genes is preferred, because these promoters may be induced by adding xylose. Pichia stipitis, when used as a host organism, exhibits the great advantage of having an efficient secretion system. This facilitates an efficient expression not only of proteins, which stay inside the cell, but also of proteins, which are continuously secreted into the medium. A further advantage of the Pichia stipitis expression system is the possibility of using xylose as a substrate. Xylose is a rather inexpensive, readily available nutrient.

35 The invention will be discussed in detail by way of the following figures and examples.

BRIEF DESCRIPTION OF THE FIGURES:

45

Fig. 1

A: restriction map of the DNA fragment encoding the xylose reductase gene (XYL1)

50 E: EcoR1, H: HindIII, B: BamHI, N: Ncol,

P: PvuII, Ps: PstI

B: restriction map of the DNA fragment encoding the xylitol dehydrogenase gene (XYL2)

55 Ba: BamHI, B: BglII, E: EcoRI, X: XbaI, S: Sall

Fig. 2

A) Nucleotide sequence of the XYL1 structural gene including its 5'- and 3'-flanking sequences and the corresponding amino acid sequence.

5 B) Nucleotide sequence of the XYL2 structural gene including its 5'- and 3'-flanking sequences and the corresponding amino acid sequence.

Fig. 3 S. cerevisiae and S. pombe expression vector. Plasmid pRD1 contains both the xylose reductase gene and xylitol dehydrogenase gene under control of their original promoters.

10 Fig. 4 Fermentation curve of PK4 grown in YNB, 2% xylose medium. The culture was inoculated with 10^8 cells/ml from a xylose grown preculture. The figure shows xylose consumption and conversion into ethanol with a theoretical maximum yield.

15 Fig. 5 (1,2) Construction scheme for constructing the vector pBRPGAM. For constructing this vector, the 3.8 kb EcoRI-PvuII-fragment from pBRSwARSGAM containing the functional GAM promoter and base pairs 1 to 208 of the coding GAM sequence was ligated to the small EcoRI-PvuII-fragment of pBR322.

20 Fig. 6 (1,2) Construction scheme for constructing the vector pBRGC1. For constructing this vector, the 3.4 kb PvuII-fragment of pCT603 containing the structural gene for xylose starting with nucleotide + 122 was inserted into the PvuII site of vector pBRPGAM.

25 Fig. 7 (1,2) Construction scheme for constructing the vector pMPGC1-2. The 6.5 kb BamHI-PstI-fragment of pBRGC1 containing the cellulase gene under control of the GAM promoter was ligated with the large BamHI-PstI-fragment of pCJD5-1.

EXAMPLES

Materials and Methods

30 I. Microorganisms and cultivation

Yeast strains:

35 1. S. cerevisiae:

- a) XJB3-1B (MAT α , met6, gal2) was obtained from the Yeast Genetic Stock Center (see Catalogue of the Yeast Genetic Stock Center, 6. edition, 1987).
- b) GRF18 (MAT α , leu2-3, leu2-112, his3-11, his3-15) was obtained from G.R. Fink (DSM 3796).
- 40 c) AH22 (MAT α , can1, his4-519, leu2-3, leu2-112) was obtained from A. Hinnen (DSM 3820).

2. Schizosaccharomyces pombe (leu1-32, his5-303) (DSM 3796).

45 3. P. stipitis CBS5773 (DSM 5855) was obtained from Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands.

50 Yeast strains were grown at 30°C in YP medium (1% yeast extract, 2% bacto pepton) or in 0.67% Difco yeast nitrogen base (YNB) without amino acids, optionally supplemented with appropriate amino acids. Media were supplied with either 2% xylose or 2% glucose. The yeasts were transformed according to Dohmen et al. (1989).

E. coli strains:

1. DH5 α F' (supplied by BRL company, Eggenstein, FRG)

55 2. HB101 (DSM 3788) (Bolivar et al., 1977).

E. coli strains were grown at 37°C in rich medium (LB-medium, Maniatis et al., 1982). The medium was supplemented with penicillin G (100 μ g/ml) when selecting for transformants. E. coli transformation was carried out as de-

scribed by Maniatis (1982).

II. Purification of the XR and XDH proteins from P. stipitis

5 Cells were grown under induced conditions to exponential growth phase. To prepare cell-free extracts cells were harvested by centrifugation and were broken with glass beads in a Braun homogenizer using 0.1 M Tris-HCl buffer (pH 7.0). The supernatant obtained following 1 h centrifugation of the crude extract ($150000 \times g$) was loaded on an affinity chromatography column (Affi-Gel Blue, 60x50 mm) preequilibrated with 5 mM NaPO₄ buffer (pH 6.8) and eluted with 1.5 mM NAD. The fractions containing XR and XDH activity were pooled and dialysed against 20 mM Tris-HCl (pH 7.5). The dialysate was subsequently applied to a DEAE-Sephadex anion exchange column preequilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted with a linear gradient (20-250 mM Tris-HCl, pH 7.5). Fractions containing the highest activity were pooled, concentrated and loaded on a SDS-PAA-gel. After running the gel was stained with 0.1 M KCl and the XR- and XDH-proteinbands were cut out, both proteins were separately eluted from the polyacrylamide gel by dialysis using 20 mM NaPO₄ (pH 8.0), 0.1% SDS; subsequently the dialysate was concentrated. All buffers contained 0.2 mM DTT (Dithiothreitol) and 0.4 mM PMSF (Phenylmethansulfonylfluoride).

III. Preparation of antisera

20 Mice were given intraperitoneal injections of 2.5 µg protein in Freund complete adjuvant. Two weeks later the same amount of protein in Freund incomplete adjuvant was injected; a third injection was administered another 2 weeks later omitting Freund adjuvant. Antiserum was harvested six weeks after the first injection.

IV. Immunoscreening

25 Antisera raised in mice against purified P. stipitis xylose reductase (XR) and xylitol dehydrogenase (XDH) protein, respectively, were used for screening the cDNA library following the procedure of Huynh et al. (1985). The antisera were diluted 10.000-fold. Bound antibodies were visualized using an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antibody, followed by a colour development reaction with the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in combination with nitro blue tetrazolium (NBT).

V. Isolation of RNA

35 All procedures were carried out at 0 to 4°C, if not indicated otherwise. All solutions and materials were sterilized if possible. P. stipitis cells were grown to midexponential phase in the presence of xylose. Yeast cells were harvested by centrifugation, washed twice with buffer 1 (20 mM NaCl, 10 mM MgCl₂, 100 mM Tris-HCl, pH 7.6) and suspended in the same buffer (1.25 mL/g cells). 1/10 volume phenol, 200 µg/ml heparin, 100 µg/ml cycloheximid and 0.4% SDS were added. Disruption of the cells was carried out by shaking with glass beads (0.45 - 0.5 mm) in a ratio of glass beads to suspension of 1:1 (v/v) in a Braun homogenizer (Braun, Melsungen). Two volumes of buffer 2 (buffer 1 containing 100 µg/ml heparin, 50 µg/ml cycloheximid, 2% SDS) were added to the homogenate, cell debris were removed by centrifugation (10000 x g, 10 min). The solution was extracted three to five times with phenol/chloroform (1:1), once with chloroform/ isoamylalcohol (24:1). The nucleic acid was precipitated by incubating the aqueous phase with 2.5 volume of ethanol in the presence of 0.2 M NaCl over night at -20°C. The precipitate was solubilized in buffer 3 (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5); SDS and LiCl were added to a final concentration of 0.1% and 4 M, respectively. The RNA was precipitated over night at +4°C. The pellet was washed twice with 70% ethanol and suspended in sterilized H₂O before use. RNA was stored at -70°C as an ethanol precipitate.

VI. Enzyme assays

50 Activities of xylose reductase (EC. 1.1.1.21) and xylitol dehydrogenase (EC. 1.1.1.9) were measured as described by Bruinenberg et al. (1983). Protein was determined with the micro biuret method according to Zamenhoff (1957) using bovine serum albumin as standard.

VII. Gelelectrophoresis

55 SDS gelelectrophoresis was carried out in 10% PAA according to Laemmli (1970).

VIII. Immunoblotting

Detection of antigenic proteins was carried out as described by Towbin et al. (1979) using the antisera obtained from mice. The proteins were transferred to a polyvinylidene difluoride microporous membran (Millipore, Immobilon PVDF) and were visualized by a phosphatase-coupled colour reaction (Blake et al., 1984). Alkaline phosphatase conjugated to goat anti-mouse IgG was obtained from Jackson Immunoresearch Lab. (Avondale, USA).

IX. DNA-sequence analysis

10 XYL1 and XYL2 genomic DNA as well as the respective cDNAs were subcloned in pT7T3-18U (Pharmacia). Fragments obtained by partial digestion using Exonuclease III (Henikoff, 1984) were analysed and sequencing was carried out by the dideoxy method of Sanger et al. (1977) using the T7-Sequencing™kit (Pharmacia). Both strands were completely determined by obtaining overlapping sequences at every junction.

15 X. Construction of a P. stipitis CBS 5773 (DSM 5855) cDNA library

Total RNA was extracted according to the method described above. Poly (A)⁺-RNA was prepared by chromatography on an oligo(dT)-cellulose column using essentially the method described by Maniatis et al. (1982). A cDNA library in λgt11 was prepared by the method of Gubler and Hoffman (1983) using a cDNA synthesis kit (Pharmacia) and in 20 vitro packaging of the recombinant λgt11-DNA according to Hohn and Murray (1974) using the in vitro packaging kit supplied by Boehringer, Mannheim (FRG).

XI. Preparation of crude extracts

25 Cells were grown to late exponential growth phase and washed twice in buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM β-mercapto ethanol). Cells were broken in an Braun homogenizer with an equal volume of glass beads. The supernatant resulting from 5 min centrifugation at 10000 g was used in enzyme assays. Extracts for Western blot analysis were boiled in 1% SDS, 5% β-mercapto ethanol, 10 mM potassium phosphate pH 7.0 and 10% glycerol.

30 EXAMPLE 1:

Isolation of the xylose reductase (XYL1) and xylitol dehydrogenase (XYL2) genes.

35 A λgt11 cDNA library constructed from poly (A)⁺-RNA of P. stipitis was screened with mouse polyclonal antibodies raised against the purified xylose reductase (XR) and xylitol dehydrogenase (XDH) proteins, respectively. Among 110.000 recombinant clones of the amplified cDNA library containing about 55.000 primary clones, seven identical XYL1 clones and three identical XYL2 clones were identified and purified. The analysis of the insert size revealed that the XYL1 clones contain two EcoR1 fragments (0.6 kb and 0.4 kb), whereas the XYL2 clones contain a single 0.55 kb 40 EcoR1 fragment. The respective EcoR1 fragments of the λgt11 clones were subcloned into the single EcoR1 site of plasmid pT7T3-18U (Pharmacia) resulting in plasmids pXRa (containing the 0.4 kb EcoR1 fragment of the XYL1 clone), pXRb (containing the 0.6 kb EcoR1 fragment of the XYL1 clone) and pXDH (containing the 0.55 kb EcoR1 fragment of the XYL2 clone).

These plasmids were used as a radioactive probe to screen a P. stipitis genomic library, which was constructed 45 by ligation of partial Sau3A digested P. stipitis DNA into the single BamH1 site of the S. cerevisiae - E. coli shuttle vector YEp13 (Broach et al., 1979) resulting in about 60.000 independent clones after transformation of E. coli HB101.

Two plasmids, namely pR1 and pD1 could be isolated and were used for transformation of S. cerevisiae GRF18. XR activity could be detected in the crude extracts of the transformants containing pR1, whereas transformants carrying 50 pD1 yielded crude extracts exhibiting XDH activity. In a mitotic stability test (Beggs 1978) the LEU2 marker and the XR or XDH gene cosegregated, indicating that pR1 and pD1 harbour the functional XYL1 (xylose reductase) and XYL2 (xylitol dehydrogenase) gene, respectively.

The plasmids pR1 and pD1 were subjected to restriction enzyme analysis yielding the map of restriction sites of the XYL1 (Fig. 1A) and XYL2 (Fig. 1B) genes, respectively.

Further subcloning experiments revealed that the XYL1 gene is encoded within a 2.04 kb BamH1 genomic fragment. 55 One of the BamH1 sites is not present in the original plasmid pR1. It must have been generated during subcloning. The XYL2 gene is encoded within a 1.95 kb BamH1-XbaI fragment. The 2.04 BamH1 fragment and the 1.95 kb BamH1-XbaI fragment were subcloned into the multiple cloning site of pT7T3-18U resulting in pR2 and pD2, respectively, and subjected to DNA sequence analysis. The DNA sequence of the structural gene and of the 5' and 3' non-coding region of

the XYL1 and the XYL2 gene is depicted in Fig. 2A and Fig. 2B, respectively.

The DNA sequence of the XYL1 gene contains an open reading frames of 954 bp (318 amino acids) whereas that of the XYL2 gene comprises an ORF of 1089 bp (363 amino acids).

The amino acids deduced from the open reading frames are shown in Fig. 2A and Fig. 2B. The sequences correspond to an XR polypeptide and an XDH polypeptide with a calculated molecular weight of 35922 and 38526 D, respectively.

EXAMPLE 2

10 Expression of both the xylose reductase and xylitol dehydrogenase gene in S. cerevisiae.

Saccharomyces cerevisiae was cotransformed with pR1 and pD1. The highest measurable activities of XR and XDH in S. cerevisiae transformed accordingly correspond to 50% of the activities of both enzymes measurable in P. stipitis crude extracts. In S. cerevisiae the genes were expressed in YNB medium containing 2% glucose as a sole carbon source, whereas in P. stipitis expression of both genes is repressed by glucose and induced by xylose. Taking into account the copy number of 10 of YEpl3 in S. cerevisiae and assuming a gene dosage dependent expression one can conclude that the Pichia promoters are 20 times less efficient in S. cerevisiae than in P. stipitis.

Furthermore, a plasmid harbouring both the XYL1 and XYL2 gene including their original Pichia promoters was constructed (Fig. 3). This plasmid pRD1 was used to transform strain GRF18 by selection on leucine resulting in the transformant PK1. However, expression was not improved compared to cotransformation with separate plasmids.

EXAMPLE 3

25 Construction of an integrative vector containing the XYL2 gene under control of different promoters

Different expression vectors using different promoters for integration and gene expression in S. cerevisiae were constructed. For example the XYL2 gene was fused to the ADH1 promoter followed by homologous integration into the HIS3 locus of S. cerevisiae. The strategy employed was as follows: The 1.5 kb XbaI/ EcoR1 fragment containing the xylitol dehydrogenase gene XYL2 was inserted into the multiple cloning site of pT7T3-18U (Pharmacia) resulting in plasmid pXDH. To eliminate the promoter region of the XYL2 gene this plasmid was linearized with XbaI (restriction site 318 bp upstream of the initiator ATG codon) and with PstI to protect the 3' end of the plasmid DNA. The linear plasmid was treated with exonuclease III and subsequently with S1 nuclease to remove the DNA between the XbaI site and the XYL2 structural gene. The deleted DNA molecules were recircularized, cloned in E. coli and the extent of deletion was determined by dideoxy sequencing. In one of the modified pXDH plasmids the 5' untranslated region and the four N-terminal amino acids were deleted. However, a new inframe ATG initiation codon was created due to the SphI site from the multiple cloning site. A BamHI linker was inserted into the HindIII site of the multiple cloning site. Subsequently, a 1.5 kb BamHI fragment carrying the XYL2 gene could be subcloned into vector pT7T3-18U resulting in additional restriction sites in front of the ATG initiation codon. The newly created 5' region is as follows: ATG CCT TGG TGT... (deletion of original amino acid 2,3 and 4).

To complete the 3' untranslated region of the XYL2 gene a 440 bp EcoRI fragment, was inserted into the single EcoRI site of the 1.5 kb fragment subcloned in pT7T3-18U. This 440 bp fragment was obtained by subcloning the 440 bp EcoRI-BamHI fragments (see Fig. 1B) into another pT7T3-18U, removing the BamHI site by cutting with BamHI and subsequent filling-in with Klenow polymerase. The 3' untranslated region could thus be isolated as 440 bp EcoRI fragment. In the single BamHI site arranged near the 5' terminus of the XYL2 gene, which is provided by the polylinker region, the 1.8 kb BamHI fragment harbouring the S. cerevisiae HIS3 gene derived from plasmid YEpl3 (Struhl et al. 1979) was inserted. To remove one of the two BamHI sites the resultant plasmid was cut with Sall and XbaI and subsequently recircularized. The resulting plasmid pXDH-HIS3 contains one suitable BamHI site in front of the ATG initiation codon in which the 1.5 kb BamHI fragment, containing the ADH1-promoter (Ammerer, 1983) of S. cerevisiae can be inserted.

Since this plasmid does not contain any autonomous replicating sequence for S. cerevisiae this plasmid can be used for homologous integration (Orr-Weaver et al. 1981) into the HIS3 locus of any S. cerevisiae strain.

In our integration experiments we used a mutagenized XJB3-1B strain called PUA6-1, which was isolated according the protocol of Porep, (1987) and Ciriacy, (1986). The resulting integrant PK2 is expressing the XYL2 gene under control of the ADH1 promoter leading to an active gene product.

EXAMPLE 4

Construction of *S. cerevisiae* and *S. pombe* Integrants expressing both the XYL1 and XYL2 gene.

5 To eliminate the promoter region of the XYL1 gene plasmid pR2 containing the XYL1 gene on a 2,04 kb BamHI fragment was linearized with XbaI (restriction site 362 bp upstream of the translation initiation ATG codon) and cleaved with SphI to protect the 3' end of the plasmid DNA. The linear plasmid was treated with exonuclease III and subsequently with S1 nuclease to remove the DNA between the XbaI site and the XYL1 structural gene. The deleted DNA molecules were recircularized, cloned in E. coli and the extent of deletion was determined by dideoxy sequencing. In one of the modified pR2 plasmids the 5' untranslated region was exactly deleted.

10 The structural gene was subcloned as a HindIII-BamHI fragment into the corresponding sites of YIp366 (Myers et al. 1986). In addition the ADH1 promoter was subcloned into the HindIII site by blunt end ligation resulting in plasmid pXR-LEU2. Since this plasmid does not contain any autonomous replicating sequence for S. cerevisiae this plasmid can be used for homologous integration (Orr-Weaver et al. 1981) into the LEU2 locus of any S. cerevisiae strain, e.g. 15 strain PK2. The resulting integrant PK3 is expressing both the XYL1 and XYL2 genes under control of the ADH1 promoter leading to active gene products. For expression studies in Schizosaccharomyces, S. pombe was transformed with both plasmids pXDH-HIS3 and pXR-LEU2 selecting for histidine and leucine. After extensive screening of the transformants for growth on xylose one transformant called AS1 could be isolated expressing both the XYL1 and XYL2 gene under control of the ADH1 promoters.

20 In the same manner other S. cerevisiae promoters, e.g. pyruvate decarboxylase (PDC) promoter (Kellermann & Hollenberg, 1988), alcoholdehydrogenase 2 (ADH2) promoter (Russell et al., 1983) or the galactokinase (GAL1/10) promoter from plasmid pBM272, which is derived from plasmid pBM150 (Johnston and Davis, 1984) by introducing a HindIII site immediately following the BamHI site, led to expression of active XYL1 and XYL2 gene product in S. cerevisiae.

25 In another set of experiments two suitable restriction sites BamHI (position -9) and Sall (position -15) were introduced just in front of the XYL1 and XYL2 genes.

30 XYL1: 5' attctttctatGTCGACGGATCCAAGATGCCTCTATT
...TAA terminator3'

35 XYL2: 5' cccctaataGTCGACGGATCCAAGATGACTGCTAAC
...TAA terminator3'

40 These modifications were introduced by site directed mutagenesis of the 5' region using the site directed mutagenesis kit supplied by Amersham according to the instructions of the manufacturer. These restriction sites offer the possibility to fuse any promoter just in front of the ATG initiation codon. In addition the gene under control of a desired promoter can be isolated as a well defined fragment for insertion into sequences suitable for homologous integration.

45 For industrial or commercial purposes it is desirable to construct stable production strains of S. cerevisiae and/or S. pombe. Therefore both genes under control of the constitutive ADH1 promoter were integrated without any bacterial sequence into the chromosome of S. cerevisiae strain PUA6-1 via homologous integration (Orr-Weaver et al. 1981). Integration into the HO homothallism gene (Russel et al. 1986), ARS-sequence (Stinchcomb et al., 1978) or into the ADH4 gene (Paquin et al., 1986) by cotransformation with pJW6 (Fogel and Welch, 1982) is preferred resulting in strains PK3(HO), PK3(ARS) and PK3(ADH4). In the case of S. pombe the integration mainly occurs via illegitimate recombination. Hence only a few of the S. pombe integrants exhibit XR and XDH activities and have the same fermentation and growth properties as the wild type.

50 The S. cerevisiae integrants PK3, PK3(HO), PK3(ARS) and PK3(ADH4) may be improved for efficient assimilation of xylulose.

EXAMPLE 5

Isolation of a S. cerevisiae mutant efficiently assimilating xylulose.

5 S. cerevisiae strain XJB3-1B grows slowly on media containing xylulose as a sole carbon source (doubling time 10 hours). According to a protocol described by Porep (Porep, 1987) a mutant, PUA3, was isolated, which utilized xylulose more efficiently than wild type S. cerevisiae strains, resulting in a doubling time of approximately four hours for growth on xylulose as a sole carbon source.

10 Mutant strain PUA3 also converts xylulose into ethanol in the absence of respiration (Porep, 1987). In order to obtain the PUA genotype in combination with an auxiliary marker (LEU2) useful in yeast transformation, strain PUA3 was crossed to AH22 (leu2 his4). From a sporulating culture of the AH22xPUA3 diploid meiotic spore progenies were isolated which were leu2 and had the ability of efficient xylulose-utilization as observed in the original mutant, PUA3. In an analogous experiment the PUA genotype was combined with leu2 and his3 auxiliary markers by crossing strain GRF18 and PUA strain and subsequent meiotic spore isolation. This resulted in strain PUA6-1 which was PUAleu2 his3.

15

EXAMPLE 6

Isolation of a S. cerevisiae mutant efficiently converting xylose into ethanol.

20

Strain PUA6-1 containing the XYL1 and XYL2 genes chromosomally integrated (See Examples 3 and 4) was able to grow on xylose as a sole carbon source whereas the untransformed PUA6-1 strain was completely negative on YNB xylose media. Doubling time of the transformant strain PK3 was 4 hours on YNB 1% xylose (for comparison, doubling time on YNB 1% glucose: 2 hours). Since ethanol production was inefficient in this strain when grown on xylose and no xylose growth was observed in the absence of respiration a mutant strain with improved capability in converting xylose to ethanol was selected as follows: 10⁸ PK3 cells were mutagenized with UV (254 nm) using conditions allowing 20% to 40% of the cells survival. The surviving cells were grown for approximately 30 generations in YNB 2% xylose liquid media. After plating on xylose solid media isolates were obtained which grow significantly faster than the parent strain PK3. One isolate was further propagated and used for selection of a mutant able to grow on YNB 2% xylose plates supplemented with 2 mg/l antimycin A in order to block respiratory metabolism. This procedure yielded a mutant (PK4) which was able to convert xylose significantly more efficiently to ethanol than the original transformant strain PK3. A typical xylose fermentation protocol is depicted in Fig. 4. The ethanol yield was approximately 40% of the initial xylose. This yield corresponds to approximately 30% of the theoretical maximum yield of ethanol from xylose conversion.

35

EXAMPLE 7

Expression of heterologous genes in Pichia stipitis

40 Following UV mutagenesis of Pichia stipitis strain CBS 5773 (DSM 5855) a trp5 mutant was isolated. The trp5 mutation was identified by examining indol accumulation according to Hagedorn and Ciriacy (Hagedorn and Ciriacy, 1969).

45 For expression in Pichia stipitis plasmids were constructed which contain a replicon from Schwanniomyces occidentalis (SwARS1), the TRP5-gene from S. cerevisiae (Dohmen et al., 1989) as a selective marker and in addition a glucoamylase(GAM)-cellulase (celD) gene fusion under control of the glucoamylase promoter. In a first step the 3.8 kb EcoRI-PvuII-fragment from plasmid pBRSwARSGAM (Fig. 5, described in EP 89 107 780) was isolated and inserted into the 2296 bp EcoRI-PvuII-fragment from pBR322 carrying the ampicillin resistance gene and the bacterial origin of replication, resulting in plasmid pBRGAM (Fig. 5). In addition to pBR322 derived sequences this plasmid carries 3.6 kb derived from the 5' noncoding region of the glucoamylase gene from Schwanniomyces occidentalis and nucleotides 1 to 208 coding for the N-terminal part including the signal sequence of the glucoamylase. Subsequently, a 3.4 kb PvuII-fragment derived from plasmid pCT603 (Joliff et al., 1986) containing the coding region of the celD-genes from Clostridium thermocellum with the exception of 120 bp (corresponding to 40 amino acids) starting with the 5' terminus of the coding region was inserted into the PvuII site of the pBRGAM resulting in pBRGC1 (Fig. 6). For construction of a P. stipitis expression vector plasmid pCJD5-1 (EP 87 110 370.1) was cleaved with BamHI/PstI and ligated with a 6.5 kb BamHI-PstI-fragment from pBRGC1. The resulting plasmid was termed pMPGC1-2 (Fig. 7). The above described P. stipitis mutant trp5 was transformed with pMPGC1-2 and the transformants were identified by their capability to grow on medium free of tryptophan (tryptophan prototrophy). Transformants were examined for cellulase activity using the congo red assay (Teather & Wood, 1982). The transformants constitutively produce active cellulase (endoglucanase

D) of Clostridium thermocellum, which is secreted into the media, indicating, that the promoter and the signal sequence encoded by the glucoamylase gene may control expression of a heterologous gene and secretion of the gene product into the medium.

Subsequently plasmid pMPGC1-2 was modified in order to substitute the glucoamylase promotor either by the S. cerevisiae ADH1-promoter or the inventive 5' regions of the XYL1 or XYL2 gene, respectively. It could be shown, that the expression under control of the XYL1 or XYL2 promoter region may be induced by xylulose, while being repressed by glucose.

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20 **Claims**

Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, GB, GR, IT, LU, NL, SE

25 1. DNA sequence, characterized in that said DNA sequence comprises a structural gene coding for a xylose reductase having the following amino acid sequence:

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	M	P	S	I	K	L	N	S	G	Y	10
5	D	M	P	A	V	G	F	G	C	W	20
10	K	V	D	V	D	T	C	S	E	Q	30
15	I	Y	R	A	I	K	T	G	Y	R	40
20	L	F	D	G	A	E	D	Y	A	N	50
25	E	K	L	V	G	A	G	V	K	K	60
30	A	I	D	E	G	I	V	K	R	E	70
35	D	L	F	L	T	S	K	L	W	N	80
40	N	Y	H	H	P	D	N	V	E	K	90
45	A	L	N	R	T	L	S	D	L	Q	100
50	V	D	Y	V	D	L	F	L	I	H	110
55	F	P	V	T	F	K	F	V	P	L	120
	E	E	K	Y	P	P	G	F	Y	C	130
	G	K	G	D	N	F	D	Y	E	D	140

	V	P	I	L	E	T	W	K	A	L	150
5											
	E	K	L	V	K	A	G	K	I	R	160
10											
	S	I	G	V	S	N	F	P	G	A	170
15											
	L	L	L	D	L	L	R	G	A	T	180
20											
	I	K	P	S	V	L	Q	V	E	H	190
25											
	H	P	Y	L	Q	Q	P	R	L	I	200
30											
	E	F	A	Q	S	R	G	I	A	V	210
35											
	T	A	Y	S	S	F	G	P	Q	S	220
40											
	F	V	E	L	N	Q	G	R	A	L	230
45											
	N	T	S	P	L	F	E	N	E	T	240
50											
	I	K	A	I	A	A	K	H	G	K	250
55											
	S	P	A	Q	V	L	L	R	W	S	260
	S	Q	R	G	I	A	I	I	P	K	270
	S	N	T	V	P	R	L	L	E	N	280

K D V N S F D L D E ²⁹⁰

5

Q D F A D I A K L D ³⁰⁰

10

I N L R F N D P W D ³¹⁰

15

W D K I P I F V * ³²⁰

20 wherein said DNA sequence is capable of expressing said polypeptide in a microorganism.

2. The DNA sequence according to claim 1, characterized in that said DNA sequence further comprises a structural gene coding for xylitol dehydrogenase having the following amino acid sequence:

25

30

35

40

45

50

55

	M	T	A	N	P	S	L	V	L	N	10
5											
	K	I	D	D	I	S	F	E	T	Y	20
10											
	D	A	P	E	I	S	E	P	T	D	30
15											
	V	L	V	Q	V	K	K	T	G	I	40
20											
	C	G	S	D	I	H	F	Y	A	H	50
25											
	G	R	I	G	N	F	V	L	T	K	60
30											
	P	M	V	L	G	H	E	S	A	G	70
35											
	T	V	V	Q	V	G	K	G	V	T	80
40											
	S	L	K	V	G	D	N	V	A	I	90
45											
	E	P	G	I	P	S	R	F	S	D	100
50											
	E	Y	K	S	G	H	Y	N	L	C	110
55											
	P	H	M	A	F	A	A	T	P	N	120
	S	K	E	G	E	P	N	P	P	G	130

	T	L	C	K	Y	F	K	S	P	E	140
5											
	D	F	L	V	K	L	P	D	H	V	150
10											
	S	L	E	L	G	A	L	V	E	P	160
15											
	L	S	V	G	V	H	A	S	K	L	170
20											
	G	S	V	A	F	G	D	Y	V	A	180
25											
	V	F	G	A	G	P	V	G	L	L	190
30											
	A	A	A	V	A	K	T	F	G	A	200
35											
	K	G	V	I	V	V	D	I	F	D	210
40											
	N	K	L	K	M	A	K	D	I	G	220
45											
	A	A	T	H	T	F	N	S	K	T	230
50											
	G	G	S	E	E	L	I	K	A	F	240
55											
	G	G	N	V	P	N	V	V	L	E	250
	C	T	G	A	E	P	C	I	K	L	260

													270
5	G	V	D	A	I	A	P	G	G	R			
	F	V	Q	V	G	N	A	A	G	P			280
10	V	S	F	P	I	T	V	F	A	M			290
15	K	E	L	T	L	F	G	S	F	R			300
20	Y	G	F	N	D	Y	K	T	A	V			310
25	G	I	F	D	T	N	Y	Q	N	G			320
30	R	E	N	A	P	I	D	F	E	Q			330
35	L	I	T	H	R	Y	K	F	K	D			340
40	A	I	E	A	Y	D	L	V	R	A			350
45	G	K	G	A	V	K	C	L	I	D			360
50	G	P	E	*									

3. The DNA sequence according to claims 1 or 2, characterized in that said DNA sequence is derived from a yeast, preferably from a yeast selected from a group consisting of the genera Schwanniomyces, Saccharomyces, Kluveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, and Pachysolen.
4. The DNA sequence according to claim 3, characterized in that the yeast is Pichia stipitis, preferably Pichia stipitis CBS 5773 (DSM 5855).
5. The DNA sequence according to claim 1, comprising the following nucleotide sequence:

-350
GGATCCACAGACACTAATTGGTTCTA

5

-310

10

-290
GTTGGCGGTTCCTGTCTGCAGTCCTCCAGC

15

-250
ACCTTCTTGCTCAACCCCCAGAAGGTGCACA

20

-230
CTGCAGACACACATACATACTCGAGAACCTGG

25

-190

30

-130

AATGGGGTATAAAATATGGCGATTCTCCG

-50
GAGAATTTCAGTTTCTTTCAATTCTC

45

CAGTATTCTTTCTATACAAC TATACTACA

50

10 ATGCCTTCTATTAAAGTTGA ACTCTGCTTA 30

50

70
AAAGTCGACGTGACACACCTGTTCTGAACAG

5

110
ATCTACCGTGCTATCAAGACC GGTTACAGA

10
TTGTTCGACGGTGCCGAAGATTACGCCAAC

15
170
GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG

20
GCCATTGACGAAGGTATCGTCAAGCGTGAA

25
230
GACTTGTTCCTTACCTCCAAGTTGTGGAAC

270
AACTACCACCAACCCAGACAACGTCGAAAAG

30
290
GCCTTGAACAGAACCCCTTCTGACTTGCAA

35
310
GTTGACTACGTTGACTTGTCTGATCCAC

330

40
350
TTCCCAGTCACCTCAAGTTGTTCCATTA

370
390
GAAGAAAAGTACCCACCAAGGATTCTACTGT

45
410
GGTAAGGGTGACAACCTCGACTACGAAGAT

430
450
GTTCCAATTTAGAGACCTGGAAAGGCTCTT

50
470
GAAAAGTTGGTCAAGGCCGGTAAGATCAGA

55

490 510
TCTATCGGTGTTCTAACCTCCCAGGTGCT

5 530
TTGCTCTTGGACTTGTTGAGAGGTGCTACC

10 550 570
ATCAAGCCATCTGTCTTGCAAGTTGAACAC

15 590
CACCCATACTTGCAACAACCAAGATTGATC

20 610 630
GAATTCGCTCAATCCCGTGGTATTGCTGTC

25 650
ACCGCTTACTCTTCGTTGGTCCTCAATCT

30 670 690
TTCGTTGAATTGAACCAAGGTAGAGCTTG

35 710
AACACTTCTCCATTGTTGGAGAACGAAACT

40 730 750
ATCAAGGCTATCGCTGCTAACGCACGGTAAG

45 770
TCTCCAGCTCAAGTCTTGAGATGGTCT

50 790 810
TCCCAAAAGAGGCATTGCCATTCCAAAAG

55 830
TCCAACACTGTCCAAAGATTGTTGGAAAC

850 870
AAGGACGTCAACAGCTTCGACTTGGACGAA

890
CAAGATTTCGCTGACATTGCCAAGTTGGAC

5 910 930
ATCAAATTGAGATTCAACGACCCATGGGAC

10 950
TGGGACAAGATTCTATCTTCGTCTAAGAA

15 970 990
GGTTGCTTATAGAGAGGAAATAAACCTA

20 1010
ATATACATTGATTGTACATTTAAAATTGAA

25 1030 1050
TATTGTAGCTAGCAGATTCGGAAATTAAA

30 1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

35 1090 1110
TCTCTATGTACATACACGTTGAAGATAGCA

40 1130
GTACAGTAGACATCAAGTCTACAGATCATT

45 1150 1170
AAACATATCTTAAATTGTAGAAAACCTATAA

50 1190
ACTTTCAATTCAAACCATGTCTGCCAAGG

55 1210 1230
AATCAAAATGAGATTTTTTCGCAGCCAAAC

60 1250
TTGAAATCCAAAAATAAAAAACGTCATTGTC

65 1270 1290
TGAAACAACTCTATCTTATCTTCACCTCA

70 1310
TCAATTCAATTGCATATCATAAAAGCCTCCG

1330 1350
ATAGCATAACAAAACCTACTTCTGCATCATAT

5

1370
CTAAATCATAGTGCCATATTCAAGTAACAAT

10

1390 1410
ACCGGTAAGAAACCTCTATTTTTAGTCT

15

1430
GCCTTAACGAGATGCAGATCGATGCAACGT

20

1450 1470
AAGATCAAACCCCTCCAGTTGTACAGTCAG

25

1490
TCATATAGTGAACACCGTACAATATGGTAT

30

1510 1530
CTACGTTCAAATAGACTCCAATACAGCTGG

35

1550
TCTGCCAAGTTGAGCAACTTTAATTAGA

40

1570 1590
GACAAAGTCGTCTCTGTTGATGTAGGCACC

45

1610
ACACATTCTCTCTGCCCGTGAACCTGT

50

1630 1650
TCTGGAGTGGAAACATCTCCAGTTGTCAA

55

1670
TATCAAACACTGACCAGGCTCAACTGGTA

1690
GAAGATTTCGTTTCGGGATC

6. The DNA sequence according to claim 2, comprising the following nucleotide sequence:

5

10

15

20

25

30

35

40

45

50

55

-310 -290
TCTAGACCACCCCTAAGTCGTCCCTATGTCG

5

-270
TATGTTGCCTCTACTACAAAGTTACTAGC

10

-250 -230
AAATATCCGCAGCAACAAACAGCTGCCCTCT

15

-210
TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

20

-190 -170
CGCTTTCGGGCTCCAGCTTCTGTCCTCTGC

25

-150
GGCTGCTGCACATAACGCGGGGACAATGAC

30

-130 -110
TTCTCCAGCTTTATTATAAAAGGAGCCAT

35

-90
CTCCTCCAGGTGAAAAATTACGATCAACTT

40

-70 -50
TTACTCTTTCCATTGTCTCTGTGTATAC

45

-30
TCACTTTAGTTGTTCAATCACCCCTAAT

50

-10 10
ACTCTTCACACAATTAAAATGACTGCTAAC

55

30
CCTTCCTTGGTGTGAACAGATCGACGAC

50 70
ATTTCGTTGAAACTTACGATGCCAGAA

90
ATCTCTGAACCTACCGATGTCCTCGTCCAG

5
110 130
GTCAAGAAAACCGGTATCTGTGGTTCCGAC

10 150
ATCCACTTCTACGCCCATGGTAGAATCGGT

15 170 190
AACTTCGTTTGACCAAGCCAATGGTCTTG

20 210
GGTCACGAATCCGCCGGTACTGTTGTCCAG

25 230 250
GTTGGTAAGGGTGTCACCTCTCTTAAGGTT

30 270
GGTGACAACGTCGCTATCGAACCAAGGTATT

35 290 310
CCATCCAGATTCTCCGACGAATAAGAGC

330
GGTCACTACAACTTGTGTCCCTCACATGGCC

40 350 370
TTCGCCGCTACTCCTAACTCCAAGGAAGGC

45 390
GAACCAAACCCACCAGGTACCTTATGTAAG

50 410 430
TACTTCAAGTCGCCAGAAGACTTCTTGGTC

55 450
AAGTTGCCAGACCACGTAGCTTGGAACTC

470 490
GGTGCTCTTGTGAGCCATTGTCTGTTGGT

5 510
GTCCACGCCTCCAAGTTGGGTTCCGTTGCT

10 530 550
TTCGGCGACTACGTTGCCGTCTTGGTGCT

15 570
GGTCCTGTTGGTCTTGGCTGCTGCTGTC

20 590 610
GCCAAGACCTTCGGTGCTAAGGGTGTCACTC

25 630
GTCGTTGACATTTGACAACAAGTTGAAG

30 650 670
ATGGCCAAGGACATTGGTGCTGCTACTCAC

35 690
ACCTTCAACTCCAAGACCCGGTGGTTCTGAA

40 710 730
GAATTGATCAAGGCTTCGGTGGTAACGTG

45 750
CCAAACGTGTTGGAAATGTACTGGTGCT

50 770 790
GAACCTTGTATCAACTTGGGTGTTGACGCC

55 810
ATTGCCCCAGGTGGTCGTTCGTTCAAGTT

830 850
GGTAACGCTGCTGGTCCAGTCAGCTTCCCA

870
ATCACCGTTTCGCCATGAAGGAATTGACT

5

890 910
TTGTTCGGTTCTTCAGATAACGGATTCAAC

10

930
GACTACAAGACTGCTGTTGGAATCTTGAC

15 970
ACTAACTACCAAAACGGTAGAGAAAATGCT

20 990
CCAATTGACTTGAACAATTGATCACCCAC

25 1010 1030
AGATACAAAGTTCAAGGACGCTATTGAAGCC

30 1050
TACGACTTGGTCAGAGCCGTAAGGGTGCT

35 1070 1090
GTCAAAGTGTCTCATTGACGGCCCTGAGTAA

40 1110
GTCAACCGCTTGGCTGGCCCCAAGTGAACC

45 1130 1150
AGAAACGAAATTGATTATCAATTAGCTTA

50 1170
TAGACCTTATCGAAATTATGTAAACTAA

55 1190 1210
TAGAAAAGACAGTGTAGAAGTTATATGGTT

55 1230
GCATCACGTGAGTTCTGAATTCTTGAAA

1250

5 GTGAAGTCTGGTCGAAACAAACAAACAAA

1270

10 AAAATATTTCAGCAAGAGTTGATTCTTT

1290

15 TCTGGAGATTTGGTAATTGACAGAGAAC

1310

1330

20 GAATAGAACTTACTGGATGGCCGCCTAGT

1350

25 GTTGAGTATATATTATCAACCAAAATCCTG

1370

1390

30 TATATAGTCTCTGAAAAATTGACTATCCT

1410

35 AACTTAACAAAAGACCACCATATGCAAGC

1430

1450

40 AGCCAAACAAATGTCCTGGCCTCTAAAG

1470

45 TCATAGTTCTTAGAGACACCAACTATACTT

1490

1510

50 AAGCATTAGCAGCTGGTCCCCAGAAGTTGC

1530

1550

55 ACAACTTCTTCATCAAGTTACCCCCAGAC

1570

1590

CGTTGCCGAATATTGGAAAAGCCTTCGA

1610

1630

CTATAGTGGATCC

7. The DNA sequence according to any of claims 1 to 6, characterized in that it is obtained by recombinant DNA technology from natural and/or cDNA and/or chemically synthesised DNA.

5 8. The combination of DNA sequences, characterized in that said combination comprises a first DNA sequence according to any of claims 1 to 7 and one or more further DNA sequences capable of regulating the expression of a structural gene encoded by said DNA sequence in a host microorganism.

10 9. The combination of DNA sequences according to claim 8, characterized in that said combination comprises modifications of the DNA sequences retaining their capability to express a functional enzyme having xylose reductase or xylitol dehydrogenase activity.

15 10. The combination of DNA sequences according to claim 8 or 9, characterized in that said structural gene contains DNA sequences derived from the structural gene coding for xylose reductase or xylitol dehydrogenase which modify said protein product while retaining its functions in such a way that said protein product is expressed as a gene product having enzymatic activity.

20 11. The combination of DNA sequences according to any of claims 8 to 10, characterized in that said DNA sequences capable of regulating the expression of said structural gene in a host microorganism are derived from said host microorganism.

12. The combination according to claim 11, characterized in that said DNA sequences capable of regulating the expression are inducible promoters.

25 13. The combination according to claim 12, characterized in that said DNA sequences capable of regulating the expression are selected from the following promoters:
ADH1, ADH2, PDC, GAL1/10.

30 14. The combination according to any of claims 11 to 13, characterized in that said DNA sequence capable of regulating the expression of said structural gene is a strong promoter, leading to over expression of the protein encoded by said structural gene.

15. A vector, characterized in that said vector comprises a DNA sequence according to any of claims 1 to 7 or a combination of DNA sequences according to any of claims 8 to 14.

35 16. The vector according to claim 15, characterized in that said vector is selected from the group comprising the plasmids pR1, pR2, pD1, pD2, pRD1, pXRa, pXRb, pXDH, pXR, pXDH-HIS3, pXR-LEU2.

40 17. A microorganism, characterized in that said microorganism is capable of expressing a xylose reductase or xylose reductase and xylitol dehydrogenase as a result of having received DNA sequences comprising the DNA sequences according to any of claims 1 to 7 or a combination of DNA sequences according to any of claims 8 to 14, coding for said xylose reductase or said xylose reductase and said xylitol dehydrogenase, by recombinant DNA technology.

45 18. The microorganism according to claim 17, characterized in that said microorganism is selected from a group consisting of yeast of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaromyces, Metschnikowia, Pachysolen, or Paecilomyces or bacteria of the genus Zy- monomas.

50 19. The microorganism according to claim 18, characterized in that said microorganism is Saccharomyces cerevisiae.

20. The microorganism according to claim 18, characterized in that said microorganism is Schizosaccharomyces pombe.

55 21. The microorganism according to any of claims 17 to 20, characterized in that said DNA sequence or combination of DNA sequences is integrated into the genome of said microorganism.

22. The microorganism according to any of claims 17 to 21, characterized in that said microorganism is useful in biomass production, in food industry and fermentation processes.

23. The microorganism according to claim 22, characterized in that said microorganism is useful for fermentation of xylose into ethanol.

5 24. A method for producing xylose reductase or xylose reductase and xylitol dehydrogenase by cultivating a microorganism according to any of claims 17 to 21 under suitable conditions and recovering said enzyme(s) in a manner known per se.

10 25. The method according to claim 24, characterized in that said microorganism is selected for efficient fermentation of xylulose.

15 26. The method according to claim 24 or 25, characterized in that said microorganism has received said DNA sequences or said combination of DNA sequences by transformation using a vector, said vector being preferably a DNA fragment or a plasmid.

20 27. The method according to claim 26, characterized in that said vector contains DNA, which is homologous to DNA of said microorganism, leading to integration into the genome of said microorganism.

28. An ethanol manufacturing process, characterized in that a microorganism according to any of claims 17 to 23 is used.

25 29. A process according to claim 28, characterized in that the fermentation process is adapted for the production of alcoholic beverages or single cell protein produced from substrates containing free xylose, preferably released by xylanase and/or xylosidase activity.

30 30. A process for production of biomass, characterized in that a host microorganism according to any of claims 17 to 23 is used.

30 **Claims for the following Contracting State : ES**

35 1. A method for preparing a DNA sequence, which DNA sequence comprises a structural gene coding for a xylose reductase having the following amino acid sequence:

40

45

50

55

M P S I K L N S G Y¹⁰
 5
 D M P A V G F G C W²⁰
 10
 K V D V D T C S E Q³⁰
 15
 I Y R A I K T G Y R⁴⁰
 20
 L F D G A E D Y A N⁵⁰
 25
 E K L V G A G V K K⁶⁰
 30
 A I D E G I V K R E⁷⁰
 35
 D L F L T S K L W N⁸⁰
 40
 N Y H H P D N V E K⁹⁰
 45
 A L N R T L S D L Q¹⁰⁰
 50
 V D Y V D L F L I H¹¹⁰
 55
 F P V T F K F V P L¹²⁰
 60
 E E K Y P P G F Y C¹³⁰
 65
 G K G D N F D Y E D¹⁴⁰

V P I L E T W K A L 150
 5
 E K L V K A G K I R 160
 10
 S I G V S N F P G A 170
 15
 L L L D L L R G A T 180
 20
 I K P S V L Q V E H 190
 25
 H P Y L Q Q P R L I 200
 30
 E F A Q S R G I A V 210
 35
 T A Y S S F G P Q S 220
 40
 F V E L N Q G R A L 230
 45
 N T S P L F E N E T 240
 50
 I K A I A A K H G K 250
 55
 S P A Q V L L R W S 260
 60
 S Q R G I A I I P K 270
 65
 S N T V P R L L E N 280

K D V N S F D . L D E 290

5

Q D F A D I A K L ³⁰⁰ D

10

I N L R F N D P W D 310

15

W D K I P I F V *

20

said DNA sequence being capable of expressing said polypeptide in a microorganism, wherein said DNA sequence is prepared by recombinant DNA technology from natural and/or cDNA and/or chemically synthesized DNA

2. A method according to claim 1, wherein said DNA sequence further comprises a structural gene encoding xylitol dehydrogenase having the following amino acid sequence:

25

30

35

40

45

50

55

M	T	A	N	P	S	L	V	L	10 N
K	I	D	D	I	S	F	E	T	20 Y
10									
D	A	P	E	I	S	E	P	T	30 D
15									
V	L	V	Q	V	K	K	T	G	40 I
20									
C	G	S	D	I	H	F	Y	A	50 H
25									
G	R	I	G	N	F	V	L	T	60 K
30									
P	M	V	L	G	H	E	S	A	70 G
35									
T	V	V	Q	V	G	K	G	V	80 T
40									
S	L	K	V	G	D	N	V	A	90 I
45									
E	P	G	I	P	S	R	F	S	100 D
50									
E	Y	K	S	G	H	Y	N	L	110 C
55									
P	H	M	A	F	A	A	T	P	120 N
60									
S	K	E	G	E	P	N	P	P	130 G

5 G V D A I A P G ²⁷⁰
 G R

10 F V Q V G N A A ²⁸⁰
 G P

15 V S F P I T V F ²⁹⁰
 A M

20 K E L T L F G S ³⁰⁰
 F R

25 Y G F N D Y K T ³¹⁰
 A V

30 R E N A P I D F ³²⁰
 E Q

35 L I T H R Y K F ³³⁰
 K D

40 A I E A Y D L V ³⁴⁰
 R A

45 G K G A V K C L ³⁵⁰
 I D

50 G P E *

55 3. The method according to any of claims 1 or 2, characterized in that said DNA sequence is derived from a yeast,
 preferably from a yeast selected from a group consisting of the genera Schwanniomyces, Saccharomyces, Kluy-
veromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, and Pachysolen.

4. The method according to claim 3, characterized in that the yeast is Pichia stipitis, preferably Pichia stipitis 5773
 (DSM 5855).

EP 0 450 430 B1

5. The method according to claim 1, wherein the DNA sequence comprises the following nucleotide sequence:

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EP 0 450 430 B1

-350

GGATCCACAGACACTAATTGGTTCTA

-310

CATTATT CGT GTTCAGACACAAACCCAGC

-290

GTTGGCGGTTCTGTCTGC GTT CCT CCAGC

-250

ACCTTCTTGCTCAACCCAGAAGGTGCACA

-230

CTGCAGACACACATAACATACGAGAACCTGG

-190

AACAAATATCGGTGTCGGTGACCGAAATGT

-170

GCAAACCCAGACACGACTAATAAACCTGGC

-130

AGCTCCAATACCGCCGACAACAGGTGAGGT

-110

GACCGATGGGTGCCATTAAATGTCTGAAA

-70

ATTGGGGTATATAAATA TGGCGATTCTCCG

-50

GAGAATT TTT CAGTTTCTTTCATTTCTC

-10

CAGTATTCTTTCTATA CAACTATACTACA

10

ATGCCTTCTATTAAAGTTGAAC TCTGGTTAC

30

GACATGCCAGCCGT CGGTT CGGCT GTT GG

50

490 510
TCTATCGGTGTTCTAACCTCCCAGGTGCT

5 530
TTGCTCTTGGACTTGTTGAGAGGTGCTACC

10 550 570
ATCAAGCCATCTGTCTTGCAAGTTGAACAC

15 590
CACCCATACTTGCAACAAACCAAGATTGATC

20 610 630
GAATTCGCTCAATCCCGTGGTATTGCTGTC

25 650
ACCGCTTACTCTTCGTTCGGTCCCTCAATCT

30 670 690
TTCGTTGAATTGAACCAAGGTAGAGCTTG

35 710
AACACTTCTCCATTGTTCGAGAACGAAACT

40 730 750
ATCAAGGCTATCGCTGCTAACGCACGGTAAG

45 770
TCTCCAGCTCAAGTCTTGGTGGAGATGGTCT

50 790 810
TCCCAAGAGGGCATGCCATCATTCAGAAG

55 830
TCCAACACGTCCCCAAGATTGTTGGAAAC

850 870
AAGGACGTCAACAGCTTCGACTTGGACGAA

890
CAAGATTTCGCTGACATTGCCAAGTTGGAC

910 930
ATCAACTGAGATTCAACGACCCATGGGAC

950 TGGGACAAGATTCTATCTTCGTCTAAGAA

10 970 990
GGTTGCTTTATAGAGAGGAAATAAAACCTA

1010
15 ATATACATTGATTGTACATTTAAAATTGAA

1030 1050
TATTGTAGCTAGCAGATTGGAAATTTAAA

20

1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

25 1090 1110
TCTCTATGTACATAACACGTTGAAGATAGCA

30 1130
GTACAGTAGACATCAAGTCTACAGATCATT

1150 1170
AAACATATCTTAAATTGTAGAAA^{ACTATAA}

1190

40

1210 1230
AATCAAAATGAGATTTTTTCGCAGCCAAAC

45 1250
TTGAAATCCAAAATAAAAACGTCATTGTC

50 1270 1290
TGAAACAACTCTATCTTATCTTCACCTCA

1310
TCAATTCAATTGCATATCATAAAAGCCTCCG

1330 1350
 ATAGCATACAAAACCTACTTCTGCATCATAT
 5
 1370
 CTAAATCATAGTGCCATATTCAAGTAACAAT
 10
 1390 1410
 ACCGGTAAGAAACCTTCTATTTTTTAGTCT
 15
 1430
 GCCTTAACGAGATGCAGATCGATGCAACGT
 20
 1450 1470
 AAGATCAAACCCCTCCAGTTGTACAGTCAG
 25
 1490
 TCATATAGTGAACACCGTACAATATGGTAT
 30
 1510 1530
 CTACGTTCAAATAGACTCCAATACAGCTGG
 35
 1550
 TCTGCCAAGTTGAGCAACTTTAATTAGA
 40
 1570 1590
 GACAAAGTCGTCTCTGTTGATGTAGGCACC
 45
 1610
 ACACATTCTCTTGCCTGTAACTCTGT
 50
 1630 1650
 TCTGGAGTGGAAACATCTCCAGTTGTCAAA
 55
 1670
 TATCAAACACTGACCAGGCTTCAACTGGTA
 1690
 GAAGATTTCGTTTCGGGATC

6. The method according to claim 2, wherein the DNA sequence comprises the following nucleotide sequence:

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55

-310 -290

TCTAGACCACCCCTAAGTCGTCCCTATGTCG

5 -270

TATGTTGCCTCTACTACAAAGTTACTAGC

10 -250 -230

AAATATCCGCAGCAACAAACAGCTGCCCTCT

15 -210

TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

20 -190 -170

CGCTTTGGGGCTCCAGCTCTGTCCCTCTGC

25 -150

GGCTGCTGCACATAACGCGGGGACAATGAC

30 -130 -110

TTCTCCAGCTTTATTATAAAAGGAGCCAT

35 -90

CTCCTCCAGGTGAAAAATTACGATCAACTT

40 -70 -50

TTACTCTTTCCATTGTCTCTGTGTATAC

45 -30

TCACTTTAGTTGTTCAATCACCCCTAAT

50 -10 10

ACTCTTCACACAAATTAAAATGACTGCTAAC

55 30

CCTTCCTTGGTGTGAACAAAGATCGACGAC

50 70

ATTTCGTCGAAACTTACGATGCCAGAA

90

ATCTCTGAACCTACCGATGTCCTCGTCCAG

110 130

GTCAAGAAAACCGGTATCTGTGGTTCCGAC

150

ATCCACTTCTACGCCCATGGTAGAATCGGT

170 190

AACTTCGTTTGACCAAGCCAATGGTCTTG

210

GGTCACGAATCCGCCGGTACTGTTGTCCAG

230 250

GTTGGTAAGGGTGTCAACCTCTCTTAAGGTT

270

GGTGACAACGTCGCTATCGAACCAAGGTATT

290 310

CCATCCAGATTCTCCGACGAATAACAAGAGC

330

GGTCACTACAACTTGTGTCTCACATGCC

350 370

TTCGCCGCTACTCCTAACTCCAAGGAAGGC

390

GAACCAAACCCACCAGGTACCTTATGTAAG

410 430

TACTTCAAGTCGCCAGAAGACTTCTGGTC

450

AAGTTGCCAGACCACGTAGCTTGGAACTC

470 490
GGTGCTCTGTTGAGCCATTGTCTGTTGGT

5 510
GTCCACGCCTCCAAGTTGGGTTCCGTTGCT

10 550
TTCGGCGACTACGTTGCCGTCTTGGTGCT

15 570
GGTCCTGTTGGTCTTTGGCTGCTGCTGTC

20 590 610
GCCAAGACCTTCGGTGCTAAGGGTGTCACTC

25 630
GTCGTTGACATTTCGACAACAAGTTGAAG

30 650 670
ATGGCCAAGGACATTGGTGCTGCTACTCAC

35 690
ACCTTCAAATCCAAGACCCGGTGGTTCTGAA

40 710 730
GAATTGATCAAGGCTTCGGTGTTAACGTG

45 750
CCAAACGTGTTGGAAATGTAATGGTGCT

50 770 790
GAACCTTGTATCAAGTTGGGTGTTGACGCC

55 810
ATTGCCCCAGGTGGTCGTTCGTTCAAGTT

55 830 850
GGTAACGCTGCTGGTCCAGTCAGCTTCCCCA

870

ATCACCGTTTCGCCATGAAGGAATTGACT

890 910

TTGTTCGGTTCTTCAGATAACGGATTCAAC

930

GA~~T~~ACTAC~~A~~AGACTGCTGTTGGAATCTTGAC

950 970

ACTAACTACCAAAACGGTAGAGAAAATGCT

990

CCAATTGACTTGAACAATTGATCACCCAC

1010 1030

AGATACAAGTTCAAGGACGCTATTGAAGCC

1050

TACGACTTGGTCAGAGCCGGTAAGGGTGCT

1070 1090

GTCAAAGTGTCTCATTGACGGCCCTGAGTAA

1110

GTCAACCGCTTGGCTGGCCC~~A~~AGTGAACC

1130 1150

AGAAACGAA~~A~~ATGATTATCA~~A~~ATAGCTTA

1170

TAGACCTTATCGAA~~TT~~TATGTAA~~ACT~~AA

1190 1210

TAGAAAAGACAGTGTAGAAGTTATATGGTT

1230

GCATCACGTGAGTTCTTGAATTCTT~~G~~AAA

1250 1270
GTGAAGTCTGGTCGGAACAAACAAACAAA

5 1290
AAAATATTTCAGCAAGAGTTGATTCTTT

10 1310 1330
TCTGGAGATTTGGTAATTGACAGAGAAC

15 1350
CCTTTCTGCTATTGCCATCTAACACATCCTT

20 1370 1390
GAATAGAACTTACTGGATGGCCGCCTAGT

25 1410
GTTGAGTATATATTATCAACCAAAATCCTG

30 1430 1450
TATATAGTCTCTGAAAAATTGACTATCCT

35 1470
AACTTAACAAAAGAGCACCATATGCAAGC

40 1490 1510
TCATAGTTCTTAGAGACACCAACTATACTT

45 1530
AGCCAAACAAAATGTCCTGGCCTCTAAAG

50 1550 1570
AAGCATTCAACCAAGCTTCCCCAGAAGTTGC

55 1590
ACAACTTCTTCATCAAGTTACCCCCAGAC

60 1610 1630
CGTTTGCCGAATATTGGAAAAGCCTTCGA

65 1650
CTATAGTGGATCC

7. A method for preparing a combination of DNA sequences, said method comprising combining a first DNA sequence obtainable according to any of claims 1 to 6 and one or more further DNA sequences capable of regulating the expression of a structural gene encoded by said DNA sequence in a host microorganism in a manner known per se.
- 5 8. The method according to claim 7, wherein said combination of sequences comprises modifications of the DNA sequences retaining their capability to express a functional enzyme having xylose reductase or xylitol dehydrogenase activity.
- 10 9. The method according to any of claims 7 or 8, wherein said structural gene contains DNA sequences derived from the structural gene coding for xylose reductase or xylitol dehydrogenase which modify said protein product while retaining its functions in such a way that said protein product is expressed as a gene product having enzymatic activity.
- 15 10. The method according to any of claims 7 to 9, wherein said DNA sequences capable of regulating the expression of said structural gene in a host microorganism are derived from said host microorganism.
11. The method according to claim 10, wherein said DNA sequences capable of regulating the expression are inducible promoters.
- 20 12. The method according to claim 11, characterized in that said DNA sequences capable of regulating the expression are selected from the following promoters:
ADH1, ADH2, PDC, GAL1/10.
- 25 13. The method according to any of claims 10 to 12, wherein said DNA sequences capable of regulating the expression of said structural gene is a strong promoter, leading to over expression of the protein encoded by said structural gene.
- 30 14. The method for preparing a vector, said method comprising inserting a DNA sequence obtainable according to any of claims 1 to 6 or a combination of DNA sequences obtainable according to any of claims 7 to 13 into a host plasmid.
15. The method according to claim 14, characterized in that it produces a vector selected from the group comprising the plasmids pR1, pR2, pD1, pD2, pRD1, pXRa, pXRb, pXDH, pXR, pXDH-HIS3, pXR-LEU2.
- 35 16. A method for preparing a microorganism being capable of expressing a xylose reductase or xylose reductase and xylitol dehydrogenase, wherein DNA sequences comprising the DNA sequences obtainable according to any of claims 1 to 6 or a combination of DNA sequences obtainable according to any of claims 7 to 13, coding for said xylose reductase or said xylose reductase and said xylitol dehydrogenase, are introduced into a host microorganism.
- 40 17. The method according to claim 16, characterized in that said host microorganism is selected from a group consisting of yeast of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen, or Paecilomyces or bacteria of the genus Zymomonas.
- 45 18. The method according to claim 17, characterized in that said microorganism is Saccharomyces cerevisiae.
19. The method according to claim 17, characterized in that said microorganism is Schizosaccharomyces pombe.
- 50 20. The method according to any of claims 16 to 19, characterized in that said DNA sequence or combination of DNA sequences is integrated into the genome of said microorganism.
21. The method according to any of claims 17 to 21, characterized in that said microorganism is useful in biomass production, in food industry and fermentation processes.
- 55 22. The method according to claim 21, characterized in that said microorganism is useful for fermentation of xylose into ethanol.

23. A method for producing xylose reductase or xylitol dehydrogenase by cultivating a microorganism obtainable according to any of claims 16 to 20 under suitable conditions and recovering said enzyme(s) in a manner known per se.

5 24. The method according to claim 23, characterized in that said microorganism is selected for efficient fermentation of xylulose.

10 25. The method according to claim 23 or 24, characterized in that said microorganism has received said DNA sequences or said combination of DNA sequences by transformation using a vector, said vector being preferably a DNA fragment or a plasmid.

15 26. The method according to claim 25, characterized in that said vector contains DNA, which is homologous to DNA of said microorganism, leading to integration into the genome of said microorganism.

20 27. An ethanol manufacturing process, characterized in that a microorganism obtainable according to any of claims 16 to 22 is used.

28. A process according to claim 27, characterized in that the fermentation process is adapted for the production of alcoholic beverages or single cell protein produced from substrates containing free xylose, preferably released by xylanase and/or xylosidase activity.

29. A process for production of biomass, characterized in that a host microorganism according to any of claims 16 to 22 is used.

25
Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, DK, FR, GB, GR, IT, LU, NL, SE

30 1. DNA-Sequenz, dadurch gekennzeichnet, daß die DNA-Sequenz ein Strukturgen umfaßt, das für eine Xylosereuktase mit der folgenden Aminosäuresequenz kodiert:

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55

EP 0 450 430 B1

M P S I K L N S G Y 10
 D M P A V G F G C W 20
 K V D V D T C S E Q 30
 I Y R A I K T G Y R 40
 L F D G A E D Y A N 50
 E K L V G A G V K K 60
 A I D E G I V K R E 70
 D L F L T S K L W N 80
 N Y H H P D N V E K 90
 A L N R T L S D L Q 100
 V D Y V D L F L I H 110
 F P V T F K F V P L 120
 E E K Y P P G F Y C 130
 G K G D N F D Y E D 140

150

V P I L E T W K A L

160

E K L V K A G K I R

170

S I G V S N F P G A

180

L L L D L L R G A T

190

I K P S V L Q V E H

200

H P Y L Q Q P R L I

210

E F A Q S R G I A V

220

T A Y S S F G P Q S

230

F V E L N Q G R A L

240

N T S P L F E N E T

250

I K A I A A K H G K

260

S P A Q V L L R W S

270

S Q R G I A I I P K

280

S N T V P R L L E N

5 K D V N S F D L D E 290

10 Q D F A D I A K L D 300

15 I N L R F N D P W D 310

20 W D K I P I F V *
wobei die DNA-Sequenz das Polypeptid in einem Mikroorganismus exprimieren kann.

25 2. DNA-Sequenz nach Anspruch 1, dadurch gekennzeichnet, daß die DNA-Sequenz weiter ein Strukturgen umfaßt, das für eine Xylitoldehydrogenase mit der folgenden Aminosäuresequenz kodiert:

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											10
5	M	T	A	N	P	S	L	V	L	N	
	K	I	D	D	I	S	F	E	T	Y	20
10	D	A	P	E	I	S	E	P	T	D	
	V	L	V	Q	V	K	K	T	G	I	30
15	C	G	S	D	I	H	F	Y	A	H	
20	G	R	I	G	N	F	V	L	T	K	40
	P	M	V	L	G	H	E	S	A	G	50
25	T	V	V	Q	V	G	K	G	V	T	
30	S	L	K	V	G	D	N	V	A	I	60
35	E	P	G	I	P	S	R	F	S	D	
40	E	Y	K	S	G	H	Y	N	L	C	70
45	P	H	M	A	F	A	A	T	P	N	
50	S	K	E	G	E	P	N	P	P	G	80
55											90
											100
											110
											120
											130

												140
5	T	L	C	K	Y	F	K	S	P	E		
	D	F	L	V	K	L	P	D	H	V		150
10	S	L	E	L	G	A	L	V	E	P		160
	L	S	V	G	V	H	A	S	K	L		170
15	G	S	V	A	F	G	D	Y	V	A		180
	V	F	G	A	G	P	V	G	L	L		190
20	A	A	A	V	A	K	T	F	G	A		200
	K	G	V	I	V	V	D	I	F	D		210
25	N	K	L	K	M	A	K	D	I	G		220
	A	A	T	H	T	F	N	S	K	T		230
30	G	G	S	E	E	L	I	K	A	F		240
	G	G	N	V	P	N	V	V	L	E		250
35	C	T	G	A	E	P	C	I	K	L		260

5	G V D A I A P G G R
10	F V Q V G N A A G P
15	V S F P I T V F A M
20	K E L T L F G S F R
25	Y G F N D Y K T A V
30	G I F D T N Y Q N G
35	R E N A P I D F E Q
40	L I T H R Y K F K D
45	A I E A Y D L V R A
50	G K G A V K C L I D
	G P E *

3. DNA-Sequenz nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß die DNA-Sequenz von einer Hefe abgeleitet ist, bevorzugt von einer Hefe, die aus der aus den Gattungen Schwanniomyces, Saccharomyces, Kluyeromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia und Pachysolen bestehenden Gruppe ausgewählt ist.
4. DNA-Sequenz nach Anspruch 3, dadurch gekennzeichnet, daß die Hefe Pichia stipitis ist, bevorzugt Pichia stipitis CBS 5773 (DSM 5855).
5. DNA-Sequenz nach Anspruch 1, umfassend die folgende Nukleotidsequenz:

-350

GGATCCACAGACACTAATTGGTTCTA

5

-310

CATTATTCGTGTTCAGACACAAACCCCAGC

10

-290

GTTGGCGGTTTCTGTCTGCGTTCCCTCCAGC

15

-250

ACCTTCTTGCTCAACCCCAGAAGGTGCACA

20

-230

CTGCAGACACACATACATACGAGAACCTGG

25

-190

AACAAATATCGGTGTCGGTGACCGAAATGT

30

-170

GCAAACCCAGACACGACTAATAAACCTGGC

35

-130

AGCTCCAATACCGCCGACAAACAGGTGAGGT

40

-110

GACCGATGGGGTGCCAATTAATGTCTGAAA

45

-70

ATTGGGGTATATAAATATGGCGATTCTCCG

50

-50

GAGAATTTTCAGTTTCTTTCTTCATTCTC

-10

CAGTATTCTTTCTACAACTATACTACA

10

30

ATGCCTTCTATTAAGTTGAACCTCTGGTTAC

50

GACATGCCAGCCGTCGGTTCGGCTGTTGG

70 90

AAAGTCGACGTCGACACCTGTTCTAACAG

5 110

ATCTACCGTGCTATCAAGACCGGTTACAGA

10 130 150

TTGTTCGACGGTGCCGAAGATTACGCCAAC

15 170

GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG

20 190 210

GCCATTGACGAAGGTATCGTCAAGCGTGAA

25 230

GACTTGTTCCTTACCTCCAAGTTGTGGAAC

30 250 270

AACTACCACCAACCCAGACAACGTCGAAAAG

35 290

GCCTTGAACAGAACCCCTTCTGACTTGCAA

40 310 330

GTTGACTACGTTGACTTGTCTGATCCAC

45 350

TTCCCAGTCACCTCAAGTTGTTCCATT

50 370 390

GAAGAAAAGTACCCACCAAGGATTCTACTGT

55 410

GGTAAGGGTGACAACCTCGACTACGAAGAT

50 430 450

GTTCCAATTAGAGACCTGGAAAGGCTCTT

55 470

GAAAAGTTGGTCAAGGCCGGTAAGATCAGA

490

TCTATCGGTGTTCTAACCTCCCAGGTGCT

510

5

530

TTGCTCTTGGACTTGTTGAGAGGTGCTACC

10

550

ATCAAGCCATCTGTCTTGCAAGTTGAACAC

570

15

590

CACCCATACTTGCACAACAACCAAGATTGATC

20

610

GAATTCGCTCAATCCCGTGGTATTGCTGTC

630

25

650

ACCGCTTACTCTCGTCGGTCCTCAATCT

30

670

TTCGTTGAATTGAACCAAGGTAGAGCTTG

690

35

710

AACACTTCTCCATTGTTCGAGAACGAAACT

40

730

ATCAAGGCTATCGCTGGTAAGCACCGTAAG

750

45

770

TCTCCAGCTCAAGTCTTGGAGATGGTCT

50

790

TCCCAAGAGGGCATGCCATCATTCCAAG

810

55

830

TCCAACACTGTCCCAAGATTGTTGGAAAC

850

AAGGACGTCAACAGCTCGACTTGGACGAA

870

890

CAAGATTTCGCTGACATTGCCAAGTTGGAC

910 930
ATCAACTTGAGATTCAACGACCCATGGGAC

950

970 **990**
GGTTGCTTTATAGAGAGGAATAAAACCTA

1010
ATATAACATTGATTGTACATTAAAAATTGAA

1030 1050
TATTGTAGCTAGCAGATTGGAAATTAAA

1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

25 1090 1110
TCTCTATGTACATAACACGTTGAAGATACCA

1130
30 GTACAGCTAGACATCAACTCTACACATGATT

1150 1170
AAACATATCTAAATTGTAAGAAAATATAA

1190

1210 1230
ATC11NTG1NTTTEETTCC1GCC1AAC

45 1250
THE UNIVERSITY OF TORONTO LIBRARIES

1270 1290
SCALAR TEST TEST TEST TEST TEST TEST

1310

1330 1350
ATAGCATACAAAACCTACTTCTGCATCATAT

5 1370
CTAAATCATAGTGCCATATTCAAGTAACAAT

10 1390 1410
ACCGGTAAGAAACCTCTATTTTTTAGTCT

15 1430
GCCTTAACGAGATGCAGATCGATGCAACGT

20 1450 1470
AAGATCAAACCCCTCCAGTTGTACAGTCAG

25 1490
TCATATAGTGAACACCGTACAATATGGTAT

30 1510 1530
CTACGTTCAAATAGACTCCAATACAGCTGG

35 1550
TCTGCCAAGTTGAGCAACTTTAATTTAGA

40 1570 1590
GACAAAGTCGTCTCTGTTGATGTAGGCACC

45 1610
ACACATTCTCTCTGCCCGTGAACCTCTGT

50 1630 1650
TCTGGAGTGGAAACATCTCCAGTTGTCAAA

55 1670
TATCAAACACTGACCAGGCTCAACTGGTA

1690
GAAGATTCGTTTCGGGATC

6. DNA-Sequenz nach Anspruch 2, umfassend die folgende Nukleotidsequenz:

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55

-310 -290
TCTAGACCACCCCTAACGTCGTCCCTATGTCG

5

-270
TATGTTGCCTCTACTACAAAGTTACTAGC

10 -250 -230
AAATATCCGCAGCAACAAACAGCTGCCCTCT

15 -210
TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

20 -190 -170
CGCTTCGGGCTCCAGCTTCTGTCCTCTGC

25 -150
GGCTGCTGCACATAACGCGGGGACAATGAC

30 -130 -110
TTCTCCAGCTTTATTATAAAAGGAGCCAT

35 -90
CTCCTCCAGGTGAAAAATTACGATCAACTT

40 -70 -50
TTACTCTTCCATTGTCTCTGTGTATAAC

45 -30
TCACTTTAGTTGTTCAATCACCCCTAAT

50 -10 10
ACTCTTCACACAATTAAAATGACTGCTAAC

55 30
CCTTCCTTGGTGTGAACAAAGATCGACGAC

55 50 70
ATTTCGTTCGAAACTTACGATGCCCCAGAA

90

ATCTCTAACCTACCGATGTCTCGTCCAG

110 130

GTCAAGAAAACCGGTATCTGTGGTTCCGAC

10 150

ATCCACTTCTACGCCCATGGTAGAATCGGT

15 170 190

AACTTCGTTTGACCAAGCCAATGGTCTTG

20 210

GGTCACGAATCCGCCGGTACTGTTGTCCAG

25 230 250

GTTGGTAAGGGTGTCAACCTCTCTTAAGGTT

30 270

GGTGACAACGTCGCTATCGAACCAAGGTATT

35 290 310

CCATCCAGATTCTCCGACGAATAAGAGC

330

GGTCACTACAACTTGTGTCTCACATGGCC

40 350 370

TTCGCCGCTACTCCTAACTCCAAGGAAGGC

45 390

GAACCAAACCCACCAGGTACCTTATGTAAG

50 410 430

TACTTCAAGTCGCCAGAACGACTTCTGGTC

55 450

AAGTTGCCAGACCACGTCAGCTTGGAACTC

470 490
GGTGCTCTGTTGAGCCATTGTCTGTTGGT

5 510
GTCCACGGCCTCCAAGTGGGTTCCGTTGCT

10 530 . 550
TTCGGCGACTACGTTGCCGTCTTGTTGGTGCT

15 570 GGT CCT GTT GGT CTT TGG CTG CTG CTG TC

590 610
GCCAAGACCTTCGGTGCTAAGGGTGTCACTC

630 GTCGTTGACATTTCGACAACAAGTTGAAG

650 ATGGCCAAGGACATTGGTGCTGCTACTCAC 670

30 69.0
ACCTTCAACTCCAAGACCGGTGGTTCTGAA

35 710 730
GAATTGATCAAGGCTTCGGTGGTAACGTG

40 750
CCAAACGTGGTTTGCAATGTAAGTGGTGCT

770 790
GAAACCTTGTATC>AGTTGGGTGTTG>CGCC

810

830 GGTAAACGCTGCTGGTCCAGTCAGCTTCCCCA 850

870
ATCACCGTTTCGCCATGAAGGAATTGACT

5
890 910
TTGTTCGGTTCTTCAGATAACGGATTCAAC

10 930
GACTACAAGACTGCTGTTGGAAATCTTGAC

15 950 970
ACTAACTACCAAAACGGTAGAGAAAATGCT

20 990
CCAATTGACTTTGAACAAATTGATCACCCAC

25 1010 1030
AGATACAAGTTCAAGGACGCTATTGAAGCC

30 1050
TACGACTTGGTCAGAGCCGGTAAGGGTGCT

35 1070 1090
GTCAAGTGTCTCATTGACGGCCCTGAGTAA

40 1110
GTCAACCGCTTGGCTGGCCAAAGTGAACC

45 1130 1150
AGAAGCGAAATTGATTATCAATTAGCTTTA

50 1170
TAGACCTTATCGAAATTATGTAAACTAA

55 1190 1210
TAGAAAAGACAGTGTAGAAGTTATGGTT

60 1230
GCATCACGTGAGTTCTGAATTCTTGAAA

1250 1270
GTGAAGTCTTGGTCGAAACAAACAAACAAA

5 1290
AAAATATTTCAGCAAGAGTTGATTCTTT

10 1310 1330
TCTGGAGATTTGGTAATTGACAGAGAACCC

15 1350
CCTTTCTGCTATTGCCATCTAACACATCCTT

20 1370 1390
GAATAGAACTTTACTGGATGGCCGCCTAGT

25 1410
GTTGAGTATATATTATCAACCAAAATCCTG

30 1430 1450
TATATAGTCTCTGAAAAATTGACTATCCT

35 1470
AACTTAACAAAAGAGCACCATAATGCAAGC

40 1490 1510
TCATAGTTCTTAGAGACACCAACTATACTT

45 1530
AGCCAAACAAAATGTCCTTGGCCTCTAAAG

50 1550 1570
AAGCATTCTAGCAGGCTCCCCAGAAGTTGC

55 1590
ACAACTTCTTCATCAAGTTACCCCCAGAC

1610 1630
CGTTGCCGAATATTGGAAAAGCCTTCGA

CTATAGTGGATCC

7. DNA-Sequenz nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß sie durch rekombinante DNA-Technologie aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten wird.
8. Kombination von DNA-Sequenzen, dadurch gekennzeichnet, daß die Kombination eine erste DNA-Sequenz gemäß einem der Ansprüche 1 bis 7 und eine oder mehrere DNA-Sequenzen umfaßt, die die Expression eines von der DNA-Sequenz kodierten Strukturgenes in einem Wirtsorganismus regulieren kann.
9. Kombination von DNA-Sequenzen nach Anspruch 8, dadurch gekennzeichnet, daß die Kombination Modifikationen der DNA-Sequenzen umfaßt, die ihre Fähigkeit zur Expression eines funktionellen Enzyms mit Xylosereduktase- oder Xylitoldehydrogenase-Aktivität aufrecht erhält.
10. Kombination von DNA-Sequenzen nach Anspruch 8 oder 9, dadurch gekennzeichnet, daß das Strukturgen DNA-Sequenzen enthält, die von dem für Xylosereduktase oder Xylitoldehydrogenase kodierenden Strukturgenen abgeleitet sind, die das Proteinprodukt modifizieren, während seine Funktionen auf eine solche Weise beibehalten werden, daß das Proteinprodukt als ein Genprodukt mit enzymatischer Aktivität exprimiert wird.
11. Kombination von DNA-Sequenzen nach einem der Ansprüche 8 bis 10, dadurch gekennzeichnet, daß die DNA-Sequenzen, die Expression des Strukturgenes in einem Wirtsorganismus regulieren können, von dem Wirtsorganismus abgeleitet sind.
12. Kombination nach Anspruch 11, dadurch gekennzeichnet, daß die DNA-Sequenzen, die die Expression regulieren können, induzierbare Promotoren sind.
13. Kombination nach Anspruch 12, dadurch gekennzeichnet, daß die DNA-Sequenzen, die die Expression regulieren können, aus den folgenden Promotoren ausgewählt sind:
ADH1, ADH2, PDC, GAL1/10.
14. Kombination nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß die DNA-Sequenz, die die Expression des Strukturgenes regulieren kann, ein starker Promotor ist, was zur Überexpression des von dem Strukturgen kodierten Proteins führt.
15. Vektor, dadurch gekennzeichnet, daß der Vektor eine DNA-Sequenz nach einem der Ansprüche 1 bis 7 oder eine Kombination von DNA-Sequenzen nach einem der Ansprüche 8 bis 14 umfaßt.
16. Vektor nach Anspruch 15, dadurch gekennzeichnet, daß der Vektor aus der Gruppe ausgewählt ist, die die Plasmide pR1, pR2, pD1, pD2, pRD1, pXRa, pXRb, pXDH, pXR, pXDH-HIS3, pXR-LEU2 umfaßt.
17. Mikroorganismus, dadurch gekennzeichnet, daß der Mikroorganismus eine Xylosereduktase oder Xylosereduktase und Xylitolhydrogenase mittels rekombinanter DNA-Technologie exprimieren kann als Ergebnis dessen, daß er DNA-Sequenzen erhalten hat, die DNA-Sequenzen nach einem der Ansprüche 1 bis 7 oder eine Kombination von DNA-Sequenzen nach einem der Ansprüche 8 bis 14 umfassen, die für die Xylosereduktase oder die Xylosereduktase und Xylitolhydrogenase kodieren.
18. Mikroorganismus nach Anspruch 17, dadurch gekennzeichnet, daß der Mikroorganismus aus einer Gruppe ausgewählt ist, die aus Hefe der Gattungen Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen oder Paecilomyces oder Bakterien der Gattung Zymomonas besteht.
19. Mikroorganismus nach Anspruch 18, dadurch gekennzeichnet, daß der Mikroorganismus Saccharomyces cerevisiae ist.
20. Mikroorganismus nach Anspruch 18, dadurch gekennzeichnet, daß der Mikroorganismus Schizosaccharomyces pombe ist.
21. Mikroorganismus nach einem der Ansprüche 17 bis 20, dadurch gekennzeichnet, daß die DNA-Sequenz oder eine Kombination von DNA-Sequenzen in das Genom des Mikroorganismus integriert ist.
22. Mikroorganismus nach einem der Ansprüche 17 bis 21, dadurch gekennzeichnet, daß der Mikroorganismus für

die Erzeugung von Biomasse, in der Nahrungsmittelindustrie oder in Fermentationsverfahren nützlich ist.

23. Mikroorganismus nach Anspruch 22, dadurch gekennzeichnet, daß der Mikroorganismus für die Fermentation von Xylose in Ethanol nützlich ist.

5 24. Verfahren zum Erzeugen von Xylosereduktase oder Xylosereduktase und Xylitoldehydrogenase durch Kultivieren eines Mikroorganismus nach einem der Ansprüche 17 bis 21 unter geeigneten Bedingungen und Gewinnen des Enzyms (der Enzyme) in an sich bekannter Weise.

10 25. Verfahren nach Anspruch 24, dadurch gekennzeichnet, daß der Mikroorganismus für eine effiziente Fermentation von Xylulose ausgewählt wird.

15 26. Verfahren nach Anspruch 24 oder 25, dadurch gekennzeichnet, daß der Mikroorganismus die DNA-Sequenzen oder die Kombination von DNA-Sequenzen durch Transformation unter Verwendung eines Vektors erhalten hat, wobei der Vektor bevorzugt ein DNA-Fragment oder ein Plasmid ist.

20 27. Verfahren nach Anspruch 26, dadurch gekennzeichnet, daß der Vektor DNA enthält, die der DNA des Mikroorganismus homolog ist, was zur Integration in das Genom des Mikroorganismus führt.

25 28. Ethanol-Herstellungsverfahren, dadurch gekennzeichnet, daß ein Mikroorganismus gemäß einem der Ansprüche 17 bis 23 verwendet wird.

29. Verfahren nach Anspruch 28, dadurch gekennzeichnet, daß das Fermentationsverfahren an die Erzeugung von alkoholischen Getränken oder Einzelzellprotein angepaßt ist, die aus Substraten erzeugt werden, die freie Xylose enthalten, die bevorzugt durch Xylanase- und/oder Xylosidase-Aktivität freigesetzt wird.

30 30. Verfahren zum Erzeugen von Biomassen, dadurch gekennzeichnet, daß ein Wirtsorganismus gemäß einem der Ansprüche 17 bis 23 verwendet wird.

30

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zum Herstellen einer DNA-Sequenz, die ein für eine Xylosereduktase mit der folgenden Aminosäuresequenz kodierendes Strukturgen umfaßt:

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	M	P	S	I	K	L	N	S	G	Y	10
5	D	M	P	A	V	G	F	G	C	W	20
10	K	V	D	V	D	T	C	S	E	Q	30
15	I	Y	R	A	I	K	T	G	Y	R	40
20	L	F	D	G	A	E	D	Y	A	N	50
25	E	K	L	V	G	A	G	V	K	K	60
30	A	I	D	E	G	I	V	K	R	E	70
35	D	L	F	L	T	S	K	L	W	N	80
40	N	Y	H	H	P	D	N	V	E	K	90
45	A	L	N	R	T	L	S	D	L	Q	100
50	V	D	Y	V	D	L	F	L	I	H	110
55	F	P	V	T	F	K	F	V	P	L	120
	E	E	K	Y	P	P	G	F	Y	C	130
	G	K	G	D	N	F	D	Y	E	D	140

150

V P I L E T W K A L

160

E K L V K A G K I R

170

S I G V S N F P G A

180

L L L D L L R G A T

190

I K P S V L Q V E H

200

H P Y L Q Q P R L I

210

E F A Q S R G I A V

220

T A Y S S F G P Q S

230

F V E L N Q G R A L

240

N T S P L F E N E T

250

I K A I A A K H G K

260

S P A Q V L L R W S

270

S Q R G I A I I P K

280

S N T V P R L L E N

55

290

K D V N S F D L D E

300

Q D F A D I A K L D

310

I N L R F N D P W D

W D K I P I F V *

wobei die DNA-Sequenz das Polypeptid in einem Mikroorganismus exprimieren kann, wobei die DNA-Sequenz mittels rekombinanter DNA-Technologie aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA hergestellt wird.

2. Verfahren nach Anspruch 1, wobei die DNA-Sequenz weiter ein für Xylitoldehydrogenase mit der folgenden Aminosäuresequenz kodierendes Strukturgen umfaßt:

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35

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50

55

M T A N P S L V L N 10
 5 K I D D I S F E T Y
 10 D A P E I S E P T D 30
 15 V L V Q V K K T G I 40
 20 C G S D I H F Y A H 50
 25 G R I G N F V L T K 60
 30 P M V L G H E S A G 70
 35 T V V Q V G K G V T 80
 40 S L K V G D N V A I 90
 45 E P G I P S R F S D 100
 50 E Y K S G H Y N L C 110
 55 P H M A F A A T P N 120
 S K E G E P N P P G 130

	T	L	C	K	Y	F	K	S	P	E	140
5											
	D	F	L	V	K	L	P	D	H	V	150
10											
	S	L	E	L	G	A	L	V	E	P	160
15											
	L	S	V	G	V	H	A	S	K	L	170
20											
	G	S	V	A	F	G	D	Y	V	A	180
25											
	V	F	G	A	G	P	V	G	L	L	190
30											
	A	A	A	V	A	K	T	F	G	A	200
35											
	K	G	V	I	V	Y	D	I	F	D	210
40											
	N	K	L	K	M	A	K	D	I	G	220
45											
	A	A	T	H	T	F	N	S	K	T	230
50											
	G	G	S	E	E	L	I	K	A	F	240
55											
	G	G	N	V	P	N	V	V	L	E	250
	C	T	G	A	E	P	C	I	K	L	260

	G	V	D	A	I	A	P	G	G	R	270
5	F	V	Q	V	G	N	A	A	G	P	280
10	V	S	F	P	I	T	V	F	A	M	290
15	K	E	L	T	L	F	G	S	F	R	300
20	Y	G	F	N	D	Y	K	T	A	V	310
25	G	I	F	D	T	N	Y	Q	N	G	320
30	R	E	N	A	P	I	D	F	E	Q	330
35	L	I	T	H	R	Y	K	F	K	D	340
40	A	I	E	A	Y	D	L	V	R	A	350
	G	K	G	A	V	K	C	L	I	D	360
45	G	P	E	*							

50 3. Verfahren nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß die DNA-Sequenz von einer Hefe abgeleitet ist, bevorzugt von einer Hefe, die aus der Gruppe ausgewählt ist, die aus den Gattungen Schwanniomyces, Saccharomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaromyces, Metschnikowia und Pachysolen besteht.

55 4. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß die Hefe Pichia stipitis ist, bevorzugt Pichia stipitis CBS 5773 (DSM 5855).

5. Verfahren nach Anspruch 1, wobei die DNA-Sequenz die folgende Nukleotidsequenz umfaßt:

5 -350
GGATCCACAGACACTAATTGGTTCTA

10 -310
CATTATT CGT GTTCAGACACAAACCCCAGC

15 -290
GTTGGCGGTTTCTGTCTGC GTT CCT CCAGC

20 -250
ACCTTCTTGCTCAACCCCAGAAGGTGCACA

25 -230
CTGCAGACACACATAACATA CGAGAACCTGG

30 -190
AACAAATATCGGTGTCGGTGACCGAAATGT

35 -170
GCAAACCCAGACACGACTAATAAACCTGGC

40 -130
AGCTCCAATACCGCCGACAACAGGTGAGGT

45 -110
GACCGATGGGGTGCCAATTAATGTCTGAAA

50 -70
ATTGGGGTATATAAAATATGGCGATTCTCCG

55 -50
GAGAATTTTCAGTTTCTTTCATTTCTC

60 -10
CAGTATTCTTTCTATACAACTATACTACA

65 10 30
ATGCCTTCTATTAAGTTGAACTCTGGTTAC

70 50
GACATGCCAGCCGTCGGTTCGGCTGTTGG

5 490 510
TCTATCGGTGTTCTAACCTCCCAGGTGCT

10 530
TTGCTCTGGACTTGTGAGAGGGTGCTACC

15 550 570
ATCAAGCCATCTGTCTGCAAGTTGAACAC

20 590
CACCCATACTTGCAACAAACCAAGATTGATC

25 610 630
GAATTCGCTCAATCCCGTGGTATTGCTGTC

30 650
ACCGCTTACTCTCGTTCGGTCCTCAATCT

35 670 690
TTCGTTGAATTGAACCAAGGTAGAGCTTG

40 710
AACACTTCTCCATTGTTGAGAACGAAACT

45 730 750
ATCAAGGCTATCGCTGCTAACGCACGGTAAG

50 770
TCTCCAGCTCAAGTCTTGTGAGATGGTCT

55 790 810
TCCCAAGAGGCCATTGCCATCATTCCAAG

60 830
TCCAAACACTGTCCCCAGATTGTTGGAAAC

65 850 870
AAGGACGTCAACAGCTTCGACTTGGACGAA

70 890
CAAGATTCGCTGACATTGCCAAGTTGGAC

910 930
ATCAACTTGAGATTCAACGACCCATGGGAC

5 950
TGGGACAAGATTCTATCTCGTCTAAGAA

10 970 990
GGTTGCTTTATAGAGAGGAAATAAACCTA

15 1010
ATATACATTGATTGTACATTAAAATTGAA

20 1030 1050
TATTGTAGCTAGCAGATTCGGAAATTAAA

25 1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

30 1090 1110
TCTCTATGTACATACACGTTGAAGATAGCA

35 1130
GTACAGTAGACATCAAGTCTACAGATCATT

40 1150 1170
AAACATATCTTAAATTGTAGAAAACCTATAA

45 1190
ACTTTCAATTCAAACCATGTCTGCCAAGG

50 1210 1230
AATCAATTGAGATTTCGCAGCCAAAC

55 1250
TTGAATCCAATAATAAAACGTCTTGTCA

59 1270 1290
TGAAACAACTCTATCTTATCTTCACCTCA

64 1310
TCAATTCATTCATATCATAAAAGCCTCCG

1330 1350
 ATAGCATAACAAAACACTTCTGCATCATAT
 5
 1370
 CTAATCATAGTGCCATATTCAAGTAACAAT
 10
 1390 1410
 ACCGGTAAGAAACCTTCTATTTTTAGTCT
 15
 1430
 GCCTAACGAGATGCAGATCGATGCAACGT
 20
 1450 1470
 AAGATCAAACCCCTCCAGTTGTACAGTCAG
 25
 1490
 TCATATAGTGAACACCGTACAATATGGTAT
 30
 1510 1530
 CTACGTTCAAATAGACTCCAATACAGCTGG
 35
 1550
 TCTGCCAAGTTGAGCAACTTAATTTAGA
 40
 1570 1590
 GACAAAGTCGTCTCTGTTGATGTAGGCACC
 45
 1610
 ACACATTCTTCTCTGCCGTGAACCTGT
 1630 1650
 TCTGGAGTGGAAACATCTCCAGTTGTCAAA
 50
 1670
 TATCAAACACTGACCAGGCTTCAACTGGTA
 55
 1690
 GAAGATTCGTTTGGGATC

EP 0 450 430 B1

6. Verfahren nach Anspruch 2, wobei die DNA-Sequenz die folgenden Nukleotidsequenz umfaßt:

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-310 -290
TCTAGACCACCCCTAAGTCGTCCCTATGTCG

5

-270
TATTTGCCTCTACTACAAAGTTACTAGC

10

-250 -230
AAATATCCGCAGCAACAAACAGCTGCCCTCT

15

-210
TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

20

-190 -170
CGCTTCGGGCTCCAGCTTCTGTCCCTCTGC

25

-150
GGCTGCTGCACATAACGGGGGACAATGAC

30

-130 -110
TTCTCCAGCTTTATTATAAAAGGAGCCAT

35

-90
CTCCTCCAGGTGAAAAATTACGATCAACTT

40

-70 -50
TTACTCTTCCATTGTCTTGTGTATAC

45

-30
TCACTTTAGTTGTTCAATCACCCCTAAT

50

-10 10
ACTCTTCACACAATTAAATGACTGCTAAC

55

30
CCTTCCTTGGTGTGAACAAAGATCGACGAC

50 70
ATTTCGTCGAAACTTACGATGCCAGAA

90

ATCTCTGAACCTACCGATGTCCTCGTCCAG

110 130

GTCAAGAAAACCGGTATCTGTGGTTCCGAC

150

ATCCACTTCTACGCCCATGCTAGAATCGGT

170 190

AACTTCGTTTGACCAAGCCAATGGTCTTG

210

GGTCACGAATCCGCCGGTACTGTTGTCCAG

230 250

GTTGGTAAGGGTGTCACCTCTCTTAAGGTT

270

GGTGACAACGTCGCTATCGAACCAAGGTATT

290 310

CCATCCAGATTCTCCGA~~C~~GAATACAAGAGC

330

GGTCACTAC~~A~~ACTTGTGTCCCTCACATGGCC

350 370

TTCGCCGCTACTCCTAACTCCAAGGAAGGC

390

GAACCAAACCCACCAGGTACCTTATGTAAG

410 430

TACTTCAAGTCGCCAGAAGACTTCTTGGTC

450

AAGTTGCCAGACCACGT~~C~~AGCTTGGAACTC

5 470 490
GGTGCTCTGTTGAGCCATTGTCTGTTGGT
10 510
GTCCACGCCTCCAAGTTGGGTTCCGTTGCT
15 530 550
TTCGGCGACTACGTTGCCGTCTTGGTGCT
20 570
GGTCCTGTTGGTCTTTGGCTGCTGCTGTC
25 590 610
GCCAAGACCTTCGGTGCTAAAGGGTGTCAAC
30 630
GTCGTTGACATTTCGACAAACAAGTTGAAG
35 650 670
ATGGCCAAGGACATTGGTGCTGCTACTCAC
40 690
ACCTTCAACTCCAAGAACCGGTGGTCTGAA
45 710 730
GAATTGATCAAGGCTTCGGTGTTAACGTG
50 750
CCAAACGGCTTTGCAATGTACTGGTGCT
55 770 790
GAACCTTGTATCAAGTTGGGTGTTAACGCC
60 810
ATTGCCCCAGGTGGTCGTTCGTTCAAGTT
65 830 850
GGTAACGCTGCTGGTCCAGTCAGCTTCCCC

5

870

ATCACCGTTTCGCCATGAAGGAATTGACT

10

890 910

TTGTTCGGTTCTTCAGATAACGGATTCAAC

15

930

GACTACAAGACTGCTGTTGGAATCTTGAC

20

950 970

ACTAACTACCAAAACGGTAGAGAAAATGCT

25

990

CCAATTGACTTGAACAATTGATCACCCAC

30

1010 1030

AGATACAAGTTCAAGGACGCTATTGAAGCC

35

1050

TACGACTTGGTCAGAGGCCGTAAGGGTGCT

40

1070 1090

GTCAAGTGTCTCATTGACGGCCCTGAGTAA

45

1110

GTCAACCGCTTGGCTGGCCCCAAGTGAACC

50

1130 1150

AGAAACGAAATGAAATCAAAATAGCTTTA

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1170

TAGACCTTATCGAAATTATGTAAACTAA

1190 1210

TAGAAAAGACAGTGTAGAAGTTATATGGTT

1230

GCATCACGTGAGTTCTTGAATTCTTGAAAA

1250 1270
 GTGAAGTCTTGGTCGGAACAAACAAACAAA
 5 1290
 AAAATATTTCAGCAAGAGTTGATTCTTT
 10 1310 1330
 TCTGGAGATTTGGTAATTGACAGAGAACCC
 15 1350
 CCTTTCTGCTATTGCCATCTAACACATCCTT
 20 1370 1390
 GAATAGAACTTTACTGGATGGCCGCCTAGT
 25 1410
 GTTGAGTATATATTATCAACCAAAATCCTG
 30 1430 1450
 TATATAGTCTCTGAAAAATTGACTATCCT
 35 1470
 AACTTAACAAAAGAGCACCATAATGCAAGC
 40 1490 1510
 TCATAGTTCTTAGAGACACCAACTATACTT
 45 1530
 AGCCAAACAAAATGTCCTGGCCTCTAAAG
 50 1550 1570
 AAGCATTCAACCAAGCTTCCCCAGAAGTTGC
 55 1590
 ACAACTTCTTCATCAAGTTACCCCCAGAC
 50 1610 1630
 CGTTGCCGAATATTGGAAAAGCCTTCGA
 CTATAGGGATCC

7. Verfahren zum Herstellen einer Kombination von DNA-Sequenzen, wobei das Verfahren das Vereinen einer ersten DNA-Sequenz, erhältlich gemäß einem der Ansprüche 1 bis 6, und einer oder mehrerer weiterer DNA-Sequenzen, die die Expression eines von der DNA-Sequenz kodierten Strukturgenes in einem Wirtsorganismus regulieren können, in an sich bekannter Weise umfaßt.

5 8. Verfahren nach Anspruch 7, wobei die Kombination von Sequenzen Modifikationen der DNA-Sequenzen umfaßt, die deren Fähigkeit zur Expression eines funktionellen Enzyms mit Xylosereduktase- oder Xylitoldehydrogenase-Aktivität aufrecht erhält.

10 9. Verfahren nach einem der Ansprüche 7 oder 8, wobei das Strukturgen DNA-Sequenzen enthält, die von dem für Xylosereduktase oder Xylitoldehydrogenase kodierenden Strukturgenen abgeleitet sind, die das Proteinprodukt modifizieren, während sie seine Funktionen auf eine solche Weise aufrecht erhalten, daß das Proteinprodukt als ein Genprodukt mit enzymatischer Aktivität exprimiert wird.

15 10. Verfahren nach einem der Ansprüche 7 bis 9, wobei die DNA-Sequenzen, die die Expression des Strukturgenes in einem Wirtsorganismus regulieren können, von dem Wirtsorganismus abgeleitet sind.

11. Verfahren nach Anspruch 10, wobei die DNA-Sequenzen, die die Expression regulieren können, induzierbare Promotoren sind.

20 12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, daß die DNA-Sequenzen, die die Expression regulieren können, aus den folgenden Promotoren ausgewählt sind:
ADH1, ADH2, PDC, GAL1/10.

25 13. Verfahren nach einem der Ansprüche 10 bis 12, wobei die DNA-Sequenzen, die die Expression des Strukturgenes regulieren können, starke Promotor sind, was zur Überexpression des von dem Strukturgenen kodierten Proteins führt.

30 14. Verfahren zum Herstellen eines Vektors, wobei das Verfahren das Insertieren einer DNA-Sequenz, die gemäß einem der Ansprüche 1 bis 6 erhältlich ist, oder einer Kombination von DNA-Sequenzen, die gemäß einem der Ansprüche 7 bis 13 erhältlich ist, in ein Wirtsplasmid umfaßt.

35 15. Verfahren nach Anspruch 14, dadurch gekennzeichnet, daß es einen Vektor erzeugt, der aus der Gruppe ausgewählt ist, die die Plasmide pR1, pR2, pD1, pD2, pRD1, pXRa, pXRb, pXDH, pXDH-HIS3, pXR-LEU2 umfaßt.

40 16. Verfahren zum Herstellen eines Mikroorganismus, der Xylosereduktase oder Xylosereduktase und Xylitolhydrogenase exprimieren kann, wobei DNA-Sequenzen, die die gemäß einem der Ansprüche 1 bis 6 erhältlichen DNA-Sequenzen oder eine Kombination von DNA-Sequenzen, die gemäß einem der Ansprüche 7 bis 13 erhältlich ist, und die für die Xylosereduktase oder die Xylosereduktase und Xylitolhydrogenase kodieren, umfassen, in einen Wirtsmikroorganismus eingeführt werden.

45 17. Verfahren nach Anspruch 16, dadurch gekennzeichnet, daß der Wirtsmikroorganismus aus einer Gruppe ausgewählt ist, die aus Hefen der Gattungen Saccharomyces, Schizosaccharomyces, Schwanniomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, Pachysolen oder Paecilomyces oder Bakterien der Gattung Zymomonas besteht.

18. Verfahren nach Anspruch 17, dadurch gekennzeichnet, daß der Mikroorganismus Saccharomyces cerevisiae ist.

50 19. Verfahren nach Anspruch 17, dadurch gekennzeichnet, daß der Mikroorganismus Schizosaccharomyces pombe ist.

20. Verfahren nach einem der Ansprüche 16 bis 19, dadurch gekennzeichnet, daß die DNA-Sequenz oder eine Kombination von DNA-Sequenzen in das Genom des Mikroorganismus integriert wird.

55 21. Verfahren nach einem der Ansprüche 17 bis 21, dadurch gekennzeichnet, daß der Mikroorganismus bei der Erzeugung von Biomasse, in der Nahrungsmittelindustrie oder bei Fermentationsverfahren nützlich ist.

22. Verfahren nach Anspruch 21, dadurch gekennzeichnet, daß der Mikroorganismus für die Fermentation von Xylose

in Ethanol nützlich ist.

23. Verfahren zum Erzeugen von Xylosereduktase oder Xylosereduktase und Xylitoldehydrogenase durch Kultivieren eines Mikroorganismus, der gemäß einem der Ansprüche 16 bis 20 erhältlich ist, unter geeigneten Bedingungen und Gewinnen des Enzyms (der Enzyme) in an sich bekannter Weise.
5
24. Verfahren nach Anspruch 23, dadurch gekennzeichnet, daß der Mikroorganismus für effiziente Fermentation von Xylulose ausgewählt wird.
- 10 25. Verfahren nach Anspruch 23 oder 24, dadurch gekennzeichnet, daß der Mikroorganismus die DNA-Sequenzen oder die Kombination von DNA-Sequenzen durch Transformation unter Verwendung eines Vektors erhalten hat, wobei der Vektor bevorzugt ein DNA-Fragment oder ein Plasmid ist.
- 15 26. Verfahren nach Anspruch 25, dadurch gekennzeichnet, daß der Vektor DNA enthält, die der DNA des Mikroorganismus homolog ist, was zur Integration in das Genom des Mikroorganismus führt.
27. Ethanol-Herstellungsverfahren, dadurch gekennzeichnet, daß ein nach einem der Ansprüche 16 bis 22 erhältlicher Mikroorganismus verwendet wird.
- 20 28. Verfahren nach Anspruch 27, dadurch gekennzeichnet, daß das Fermentationsverfahren an die Erzeugung von alkoholischen Getränken oder Einzelzellprotein angepaßt ist, die aus Substraten erzeugt werden, die freie Xylose enthalten, die bevorzugt durch Xylanase und/oder Xylosidase-Aktivität freigesetzt wird.
- 25 29. Verfahren zum Erzeugen von Biomasse, dadurch gekennzeichnet, daß ein Wirtsorganismus nach einem der Ansprüche 16 bis 22 verwendet wird.

Revendications

30 Revendications pour les Etats contractants suivants : AT, BE, CH, LI, DE, DK, FR, GB, GR, IT, LU, NL, SE

1. Séquence d'ADN, caractérisée en ce que ladite séquence d'ADN comprend un gène de structure codant pour une réductase du xylose ayant la séquence suivante d'acides aminés:
35

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50

55

5 M P S I K L N S G Y 10
D M P A V G F G C W 20
10 K V D V D T C S E Q 30
I Y R A I K T G Y R 40
L F D G A E D Y A N 50
E K L V G A G V K K 60
25 A I D E G I V K R E 70
D L F L T S K L W N 80
30 N Y H H P D N V E K 90
A L N R T L S D L Q 100
35 V D Y V D L F L I H 110
F P V T F K F V P L 120
40 E E K Y P P G F Y C 130
G K G D N F D Y E D 140
45 50

150

V	P	I	L	E	T	W	K	A	L
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160

E	K	L	V	K	A	G	K	I	R
---	---	---	---	---	---	---	---	---	---

170

S	I	G	V	S	N	F	P	G	A
---	---	---	---	---	---	---	---	---	---

180

L	L	L	D	L	L	R	G	A	T
---	---	---	---	---	---	---	---	---	---

190

I	K	P	S	V	L	Q	V	E	H
---	---	---	---	---	---	---	---	---	---

200

H	P	Y	L	Q	Q	P	R	L	I
---	---	---	---	---	---	---	---	---	---

210

E	F	A	Q	S	R	G	I	A	V
---	---	---	---	---	---	---	---	---	---

220

T	A	Y	S	S	F	G	P	Q	S
---	---	---	---	---	---	---	---	---	---

230

F	V	E	L	N	Q	G	R	A	L
---	---	---	---	---	---	---	---	---	---

240

N	T	S	P	L	F	E	N	E	T
---	---	---	---	---	---	---	---	---	---

250

I	K	A	I	A	A	K	H	G	K
---	---	---	---	---	---	---	---	---	---

260

S	P	A	Q	V	L	L	R	W	S
---	---	---	---	---	---	---	---	---	---

270

S	Q	R	G	I	A	I	I	P	K
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280

S	N	T	V	P	R	L	L	E	N
---	---	---	---	---	---	---	---	---	---

K D V N S F D L D E **290**

5

O P F A D I A K L D 300

10

I N L R F N D P W D 310

15

W D K T P I E V *

dans laquelle ladite séquence d'ADN est capable d'exprimer ledit polypeptide dans un micro-organisme

20 2. Séquence d'ADN selon la revendication 1, caractérisée en ce que ladite séquence d'ADN comprend en outre un gène de structure codant pour la déshydrogénase du xylitol avant la séquence suivante d'acides aminés :

25

30

35

40

45

50

55

M T A N P S L V L N 10
 5 K I D D I S F E T Y
 10 D A P E I S E P T D 30
 15 V L V Q V K K T G I 40
 20 C G S D I H F Y A H 50
 25 G R I G N F V L T K 60
 30 P M V L G H E S A G 70
 35 T V V Q V G K G V T 80
 40 S L K V G D N V A I 90
 45 E P G I P S R F S D 100
 50 E Y K S G H Y N L C 110
 55 P H M A F A A T P N 120
 60 S K E G E P N P P G 130

50

55

140

T	L	C	K	Y	F	K	S	P	E
---	---	---	---	---	---	---	---	---	---

150

D	F	L	V	K	L	P	D	H	V
---	---	---	---	---	---	---	---	---	---

160

S	L	E	L	G	A	L	V	E	P
---	---	---	---	---	---	---	---	---	---

170

L	S	V	G	V	H	A	S	K	L
---	---	---	---	---	---	---	---	---	---

180

G	S	V	A	F	G	D	Y	V	A
---	---	---	---	---	---	---	---	---	---

190

V	F	G	A	G	P	V	G	L	L
---	---	---	---	---	---	---	---	---	---

200

A	A	A	V	A	K	T	F	G	A
---	---	---	---	---	---	---	---	---	---

210

K	G	V	I	V	V	D	I	F	D
---	---	---	---	---	---	---	---	---	---

220

N	K	L	K	M	A	K	D	I	G
---	---	---	---	---	---	---	---	---	---

230

A	A	T	H	T	F	N	S	K	T
---	---	---	---	---	---	---	---	---	---

240

G	G	S	E	E	L	I	K	A	F
---	---	---	---	---	---	---	---	---	---

250

G	G	N	V	P	N	V	V	L	E
---	---	---	---	---	---	---	---	---	---

260

C	T	G	A	E	P	C	I	K	L
---	---	---	---	---	---	---	---	---	---

50

55

270
G V D A I A P G G R
5

280
F V Q V G N A A G P
10

290
V S F P I T V F A M
15

300
K E L T L F G S F R
20

310
Y G F N D Y K T A V
25

320
G I F D T N Y Q N G
30

330
R E N A P I D F E Q
35

340
L I T H R Y K F K D
40

350
A I E A Y D L V R A
45

360
G K G A V K C L I D
50

G P E *

3. Séquence d'ADN selon les revendications 1 ou 2, caractérisée en ce que ladite séquence d'ADN est dérivée d'une levure, de préférence d'une levure choisie parmi un groupe constitué des genres Schwanniomyces, Saccharomyces, Kluyveromyces, Pichia, Hansenula, Candida, Debaryomyces, Metschnikowia, et Pachysolen.
4. Séquence d'ADN selon la revendication 3, caractérisée en ce que la levure est Pichia stipitis, de préférence Pichia stipitis CBS 5773 (DSM 5855).
5. Séquence d'ADN selon la revendication 1, comprenant la séquence suivante de nucléotides :

-350
GGATCCACAGACACTAATTGGTTCTA

-310

-290 GTTGGCGGTTCTGTCTGCAGTCCTCCAGC

-250

-230
CTGCAGACACACATACATAACGAGAACCTGG

-190

-170
GCAAACCCAGACACCGACTAATAAACCTGGC

-130

-110
GACCGATGGGGTGCCAATTATGTCTGAAA

-70

-50
GAGAATTTCTCACTTTCATTCA

-10

ATGCCTTCTATTAAAGTTGAACTCTGGTTAC

50

5 70 90
AAAGTCGACGTCGACACCTGTTCTGAACAG
10 110
ATCTACCGTGCTATCAAGACCGGTTACAGA
15 130 150
TTGTTCGACGGTGCCGAAGATTACGCCAAC
20 170
GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG
25 190 210
GCCATTGACGAAGGTATCGTCAAGCGTGAA
30 230
GACTTGTTCCTTACCTCCAAGTTGTGGAAC
35 250 270
AACTACCACCACCCAGACAACTCGAAAAG
40 290
GCCTTGAACAGAACCCCTTCTGACTTGCAA
45 310 330
GTTGACTACGTTGACTTGTCTTGATCCAC
50 350
TTCCCAGTCACCTTCAAGTTCGTTCCATT
55 370 390
GAAGAAAAGTACCCACCCAGGATTCTACTGT
60 410
GGTAAGGGTGACAACCTCCACTACGAAGAT
65 430 450
GTTCCAATTTAGAGACCTGGAAAGGCTCTT
70 470
GAAAAGTTGGTCAAGGCCGGTAAGATCAGA

5 490 510
TCTATCGGTGTTCTAACCTCCCAGGTGCT

10 530 550 570
TTGCTCTTGGACTTGTTGAGAGGTGCTACC
ATCAAGCCATCTGTCTTGCAAGTTGAACAC

15 590 610 630
CACCCATACTTGCAACAAACCAAGATTGATC
GAATTCGCTCAATCCCGTGGTATTGCTGTC

20 650 670 690
ACCGCTTACTCTTCGTTGGTCCTCAATCT
TTCGTTGAATTGAACCAAGGTAGAGCTTG

25 710 730 750
AACACTTCTCCATTGTTCGAGAACGAAACT
ATCAAGGCTATCGCTGCTAACGCACGGTAAG

30 770 790 810
TCTCCAGCTCAAGTCTTGTTGAGATGGTCT
TCCCCAAGGGCATGCCATCAATTCCAAAAG

35 830 850 870
TCCAAACACTGTCCCAGATTGTTGGAAAAC
AAGGACGTCAACAGCTTCGACTTGGACGAA

40 890 910 930
CAAGATTTCGCTGACATTGCCAAGTTGGAC

5 910 930
ATCAACTTGAGATTCAACGACCCATGGGAC

10 950
TGGGACAAGATTCTATCTTCGTCTAAGAA

15 970 990
GGTTGCTTTATAGAGAGGAAATAAACCTA

20 1010
ATATACATTGATTGTACATTAAAATTGAA

25 1030 1050
TATTGTAGCTAGCAGATTCGGAAATTAAA

30 1070
ATGGGAAGGTGATTCTATCCGTACGAATGA

35 1090 1110
TCTCTATGTACATACACGTTGAAGATAGCA

40 1130
GTACAGTAGACATCAAGTCTACAGATCATT

45 1150 1170
AAACATATCTAAATTGTAGAAAACATAAA

50 1190
ACTTTCAATTCAAACCATGTCTGCCAAGG

55 1210 1230
AATCAATTGAGATTTTTTGCAGCCAAAC

60 1250
TTGAAATCCAAAATAAAAAACGTCATTGTC

65 1270 1290
TGAAAACAATCTATCTTATCTTCACCTCA

70 1310
TCAATTCAATTGCATATCATAAAAGCCTCCG

1330 1350
ATAGCATACAAAACCTACTTCTGCATCATAT

5 1370
CTAAATCATAGTGCCATATTCAAGTAACAAT

10 1390 1410
ACCGGTAAGAAACCTCTATTTTTTAGTCT

15 1430
GCCTTAACGAGATGCAGATCGATGCAACGT

20 1450 1470
AAGATCAAACCCCTCCAGTTGTACAGTCAG

25 1490
TCATATAGTGAACACCGTACAATATGGTAT

30 1510 1530
CTACGTTCAAATAGACTCCAATACAGCTGG

35 1550
TCTGCCAAGTTGAGCAACTTAATTAGA

40 1570 1590
GACAAAGTCGTCTCTGTTGATGTAGGCACC

45 1610
ACACATTCTTCTCTGCCGTGAACTCTGT

50 1630 1650
TCTGGAGTGGAAACATCTCCAGTTGTCAA

55 1670
TATCAAACACTGACCAGGCTCAACTGGTA

60 1690
GAAGATTCTGTTTCGGGATC

6. Séquence d'ADN selon la revendication 2, comprenant la séquence suivante de nucléotides :

5

-310 -290
TCTAGACCACCCCTAACGTCGTCCCTATGTCG

10

-270
TATGTTGCCTCTACTACAAAGTTACTAGC

20

-250 -230
AAATATCCGCAGCAACAACAGCTGCCCTCT

15

-210
TCCAGCTTCTTAGTGTGGCCGAAAAGG

-190 -170
CGCTTCGGGCTCCAGCTTCTGTCCCTCTGC

-150
GGCTGCTGCACATAACGCGGGGACAATGAC

25

-130 -110
TTCTCCAGCTTTATTATAAAAGGAGCCAT

30

-90
CTCCTCCAGGTGA~~A~~AAATTACGATCAACTT

35

-70 -50
TTACTCTTCCATTGTCTCTGTGTATAAC

40

-30
TCACTTTAGTTGTTCAATCACCCCTAAT

45

-10 10
ACTCTTCACACAAATTAAAATGACTGCTAAC

30
CCTTCCTTGGTGTGAACAAAGATCGACGAC

50

50 70
ATTCGTTGAAACTTACGATGCCCGAGAA

55

90
ATCTCTGAAACCTACCGATGTCCTCGTCCAG

110
GTCAAGAAAACCGGTATCTGTGGTTCCGAC

130

150
ATCCACTTCTACGCCATGGTAGAATCGGT

170
AACTTCGTTTGACCAAGCCAATGGTCTTG

190

210
GGTCACGAATCCGCCGGTACTGTTGTCCAG

230
GTTGGTAAGGGTGTCACCTCTCTTAAGGTT

250

270
GGTGACAACGTCGCTATCGAACCAAGGTATT

290
CCATCCAGATTCTCCGACGAATAACAAGAGC

310

330
GGTCACTACAACTTGTGTCCCTCACATGGCC

35

350
TTCGCCGCTACTCCTAACTCCAAGGAAGGC

370

390
GAACCAAACCCACCAGGTACCTTATGTAAG

410
TACTTCAAGTCGCCAGAAGACTTCTGGTC

430

450
AAGTTGCCAGACCACGTCAAGCTTGGAACTC

50

5 470 490
GGTGCTCTTGTGAGCCATTGTCTGTTGGT

10 510
GTCCACGCCCTCCAAGTTGGGTTCCGTTGCT

15 530 550
TTCGGCGACTACGTTGCCGTCTTGTTGCT

20 570
GGTCCTGTTGGTCTTGGCTGCTGCTGTC

25 590 610
GCCAAGACCTTCGGTGCTAAGGGTGTCA
T

30 630
GTCGTTGACATTTCGACAACAAGTTGAAG

35 650 670
ATGGCCAAGGACATTGGTGCTGCTACTCAC

40 690
ACCTTCAACTCCAAGACCCGGTGGTCTGAA

45 710 730
GAATTGATCAGGCTTTCGGTGGTAACGTG

50 750
CCAAACGTGGTTTGGTAACTACTCGTGCT

55 770 790
GAACCTTGTATCAGTTGGGTGTTGACGCC

60 810
ATTGCCCCAGGTGGTCGTTCTGTTCAAGTT

65 830 850
GGTAACGCTGCTGGTCCAGTCAGCTTCCCCA

870
ATCACCGTTTCGCCATGAAGGAATTGACT

5
890 910
TTGTTCGGTTCTTCAGATAACGGATTCAAC

10 930
GACTACAAGACTGCTGTTGGAATCTTGAC

15 950 970
ACTAACTACCAAAACGGTAGAGAAAATGCT

20 990
CCAATTGACTTGAACAATTGATCACCCAC

25 1010 1030
AGATAACAAGTTCAAGGACGCTATTGAAGCC

30 1050
TACGACTTGGTCAGAGCCGGTAAGGGTGCT

35 1070 1090
GTCAAAGTGTCTCATTGACGGCCCTGAGTAA

40 1110
GTCAAACCGCTTGGCTGGCCCCAAAGTGAACC

45 1130 1150
AGAAGGAAATGATGATTCAGTAACTAA

50 1170
TAGACCTTATCGAAATTATGAAACTAA

55 1190 1210
TAGAAAAGAACAGTGTAGAAGTTATATGGTT

60 1230
GCATCACGTGAGTTCTTGAATTCTTGAAA

5

1250
GTGAAGTCTGGTCGAAACAAACAAACAAA

10

1290
AAAATATTTCAAGCAAGAGTTGATTCTTT

20

1310
TCTGGAGATTTGGTAATTGACAGAGAACCC

25

1350
CCTTTCTGCTATTGCCATCTAAACATCCTT

1370
GAATAGAACTTACTGGATGGCCGCCTAGT

30

1410
GTTGAGTATATATTATCAACCAAAATCCTG

35

1430
TATATAGTCTCTGAAAAATTGACTATCCT

40

1470
AACTTAACAAAAGAGCACCATATGCAAGC

45

1490
TCATAGTTCTTAGAGACACCAACTATACTT

1530
AGCCAAACAAAATGTCCTTGGCCTCTAAAG

50

1550
AAGCATTCAAGCAGCTTCCCCAGAAGTTGC

55

1590
ACACTTCTTCATCAAGTTACCCCCAGAC

60

1610
CGTTTGCCGAATATTCGGAAAGCCTTCGA

CTATAGTGGATCC

7. Séquence d'ADN selon l'une quelconque des revendications 1 à 6, caractérisée en ce qu'elle est obtenue par la technologie de l'ADN recombinant, à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé chimiquement.
8. Combinaison de séquences d'ADN, caractérisée en ce que ladite combinaison comprend une première séquence

d'ADN selon l'une quelconque des revendications 1 à 7 et une ou plusieurs autres séquences d'ADN capable de réguler l'expression d'un gène de structure encodé par ladite séquence d'ADN dans un micro-organisme hôte.

- 5 9. Combinaison de séquences d'ADN selon la revendication 8, caractérisée en ce que ladite combinaison comprend des modifications des séquences d'ADN conservant leur capacité à exprimer une enzyme fonctionnelle ayant une activité de réductase du xylose ou de déshydrogénase du xylitol.
- 10 10. Combinaison de séquences d'ADN selon la Revendication 8 ou 9, caractérisée en ce que ledit gène de structure contient des séquences d'ADN dérivées du gène de structure codant pour la réductase du xylose ou la déshydrogénase du xylitol qui modifie ledit produit de protéine tout en conservant ses fonctions d'une façon telle que ledit produit de protéine est exprimé comme un produit de gène ayant une activité enzymatique.
- 15 11. Combinaison de séquences d'ADN selon l'une quelconque des revendications 8 à 10, caractérisée en ce que lesdites séquences d'ADN capables de réguler l'expression dudit gène de structure dans un micro-organisme hôte, sont dérivées dudit micro-organisme hôte.
- 20 12. Combinaison selon la revendication 11, caractérisé en ce que lesdites séquences d'ADN capables de réguler l'expression sont des promoteurs susceptibles d'être induits.
- 25 13. Combinaison selon la revendication 12, caractérisée en ce que lesdites séquences d'ADN capables de réguler l'expression sont choisies parmi les promoteurs suivants : ADH1, ADH2, PDC, GAL1/10.
- 30 14. Combinaison selon l'une quelconque des revendications 11 à 13, caractérisée en ce que ladite séquence d'ADN capable de réguler l'expression dudit gène de structure, est un promoteur fort, conduisant à une surexpression de la protéine encodée par ledit gène de structure.
- 35 15. Vecteur, caractérisé en ce que ledit vecteur comprend une séquence d'ADN selon l'une quelconque des revendications 1 à 7 ou une combinaison de séquences d'ADN selon l'une quelconque des revendications 8 à 14.
- 40 16. Vecteur selon la revendication 15, caractérisé en ce que ledit vecteur est choisi parmi le groupe comprenant les plasmides pR1, pR2, pD1, pD2, pRD1, pXRa, pXRb, pXDH, pXR, pXDH-HIS3, pXR-LEU2.
- 45 17. Micro-organisme, caractérisé en ce que ledit micro-organisme est capable d'exprimer une réductase du xylose ou une réductase du xylose et une deshydrogénase du xylitol après avoir reçu des séquences d'ADN comprenant les séquences d'ADN selon l'une quelconque des revendications 1 à 7 ou une combinaison de séquences d'ADN selon l'une quelconque des revendications 8 à 14, codant pour ladite réductase du xylose ou ladite réductase du xylose et ladite deshydrogénase du xylitol, par la technologie de l'ADN recombinant.
- 50 18. Micro-organisme selon la revendication 17, caractérisé en ce que ledit micro-organisme est choisi parmi un groupe constitué de levures des genres *Saccharomyces*, *Schizosaccharomyces*, *Schwanniomyces*, *Kluyveromyces*, *Pichia*, *Hansenula*, *Candida*, *Debaryomyces*, *Metschnikowia*, *Pachysolen* ou *Paecilomyces* ou de bactéries du genre *Zymomonas*.
- 55 19. Micro-organisme selon la revendication 18, caractérisé en ce que ledit micro-organisme est *Saccharomyces cerevisiae*.
- 20. Micro-organisme selon la revendication 18, caractérisé en ce que ledit micro-organisme est *Schizosaccharomyces pombe*.
- 21. Micro-organisme selon l'une quelconque des revendications 17 à 20, caractérisé en ce que ladite séquence d'ADN ou la combinaison de séquences d'ADN est intégrée dans le génome dudit micro-organisme.
- 22. Micro-organisme selon l'une quelconque des revendications 17 à 21, caractérisé en ce que ledit micro-organisme est utile dans la production de biomasse, dans l'industrie alimentaire et dans les procédés de fermentation.
- 23. Micro-organisme selon la revendication 22, caractérisé en ce que ledit micro-organisme est utile pour la fermentation du xylose dans l'éthanol.

24. Procédé de production de la réductase du xylose ou de la réductase du xylose et de la déshydrogénase du xylitol par culture d'un micro-organisme selon l'une quelconque des revendications 17 à 21 sous des conditions appropriées et récupération du(des)dits enzyme(s) d'une manière connue en soi.

5 25. Procédé selon la revendication 24, caractérisé en ce que ledit micro-organisme est choisi pour la fermentation efficace du xylulose.

26. Procédé selon la revendication 24 ou 25, caractérisé en ce que ledit micro-organisme a reçu lesdites séquences d'ADN ou ladite combinaison de séquences d'ADN par transformation utilisant un vecteur, ledit vecteur étant de préférence un fragment d'ADN ou un plasmide.

10 27. Procédé selon la revendication 26, caractérisé en ce que ledit vecteur contient de l'ADN, qui est homologue à l'ADN dudit micro-organisme, conduisant à l'intégration dans le génome dudit micro-organisme.

15 28. Procédé de fabrication d'éthanol, caractérisé en ce qu'on utilise un micro-organisme selon l'une quelconque des revendications 17 à 23.

29. Procédé selon la revendication 28, caractérisé en ce que le procédé de fermentation est adapté à la production de boissons alcoolisées ou d'une protéine de cellule unique produite à partir de substrats contenant du xylose libre, de préférence libéré par l'activité de la xylanase et/ou de la xylosidase.

20 30. Procédé pour la production de biomasse, caractérisé en ce que l'on utilise le micro-organisme hôte selon l'une quelconque des revendications 17 à 23.

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Revendications pour l'Etat contractant suivant : ES

1. Procédé de préparation d'une séquence d'ADN, cette séquence d'ADN comprenant un gène de structure codant pour une réductase du xylose ayant la séquence suivante d'acides aminés :

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50

55

M P S I K L N S G Y 10
 D M P A V G F G C W 20
 K V D V D T C S E Q 30
 I Y R A I K T G Y R 40
 L F D G A E D Y A N 50
 E K L V G A G V K K 60
 A I D E G I V K R E 70
 D L F L T S K L W N 80
 N Y H H P D N V E K 90
 A L N R T L S D L Q 100
 V D Y V D L F L I H 110
 F P V T F K F V P L 120
 E E K Y P P G F Y C 130
 G K G D N F D Y E D 140

5 V P I L E T W K A L
150
E K L V K A G K I R
10 S I G V S N F P G A
170
L L L D L L R G A T
180
I K P S V L Q V E H
20 -
200 H P Y L Q Q P R L I
25 E F A Q S R G I A V
210
T A Y S S F G P Q S
30
220
F V E L N Q G R A L
35
230
N T S P L F E N E T
40
240
I X A I A A K H G K
45
250
S P A Q V L L R W S
50
260
S Q R G I A I E P K
270
S N T V P R L L E N
280

5 K D V N S F D L D E 290

Q D F A D I A K L D 300

10 I N L R F N D P W D 310

15 W D K I P I F V *

20 ladite séquence d'ADN étant capable d'exprimer ledit polypeptide dans un micro-organisme, dans lequel ladite séquence d'ADN est préparée par la technologie de l'ADN recombinant, à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé chimiquement.

25 2. Procédé selon la revendication 1, dans lequel ladite séquence d'ADN comprend en outre un gène de structure encodant pour la déshydrogénase du xylitol ayant la séquence suivante d'acides aminés :

30

35

40

45

50

55

M T A N P S L V L N ¹⁰
 5 K I D D I S F E T Y
 10 D A P E I S E P T D ³⁰
 15 V L V Q V K K T G I ⁴⁰
 20 C G S D I H F Y A H ⁵⁰
 25 G R I G N F V L T K ⁶⁰
 30 P M V L G H E S A G ⁷⁰
 35 T V V Q V G K G V T ⁸⁰
 40 S L K V G D N V A I ⁹⁰
 45 E P G I P S R F S D ¹⁰⁰
 50 E Y K S G H Y N L C ¹¹⁰
 55 P H M A F A A T P N ¹²⁰
 60 S K E G E P N P P G ¹³⁰

T L C K Y F K S P E ¹⁴⁰
 5

D F L V K L P D H V ¹⁵⁰
 10

S L E L G A L V E P ¹⁶⁰
 15

L S V G V H A S K L ¹⁷⁰
 20

G S V A F G D Y V A ¹⁸⁰
 25

V F G A G P V G L L ¹⁹⁰
 30

A A A V A K T F G A ²⁰⁰
 35

K G V I V V D I F D ²¹⁰
 40

N K L K M A .K D I G ²²⁰
 45

A A T H T F K S K T ²³⁰
 50

G G S E E L I K A F ²⁴⁰
 55

G G N V P N V V L E ²⁵⁰
 60

C T G A E P C I K L ²⁶⁰

270

5 G V D A I A P G G R

280

10 F V Q V G N A A G P

290

15 V S F P I T V F A M

300

20 K E L T L F G S F R

310

25 Y G F N D Y K T A V

320

30 G I F D T N Y Q N G

330

35 R E N A P I D F E Q

340

40 L I T H R Y K F K D

350

45 A I E A Y D L V R A

360

50 G K G A V K C L I D

G P E *

45 3. Procédé selon l'une quelconque des revendications 1 ou 2, caractérisé en ce que ladite séquence d'ADN est dérivée d'une levure, de préférence d'une levure choisie parmi un groupe constitué des genres *Schwanniomyces*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Hansenula*, *Candida*, *Debaryomyces*, *Metschnikowia*, et *Pachysolen*.

50 4. Procédé selon la revendication 3, caractérisé en ce que la levure est *Pichia stipitis*, de préférence *Pichia stipitis* 5773 (DSM 5855).

55 5. Procédé selon la revendication 1, dans lequel la séquence d'ADN comprend la séquence suivante de nucléotides :

-350

5 GGATCCACAGACACTAATTGGTTCTA

-310

10 CATTATTCGTGTTCAGACACAAACCCCAAGC

-290

15 GTTGGCGGTTCTGTCTGCCTCCAGC

-250

19 ACCTTCTTGCTCAACCCCAGAAGGTGCACA

-230

20 CTGCAGACACACATACATACTACGAGAACCTGG

-190

25 AACAAATATCGGTGTCGGTGACCGAAATGT

-170

30 GCAAACCCAGACACGACTAATAAACCTGGC

-130

35 AGCTCCAATACCGCCGACAACAGGTGAGGT

-110

40 GACCGATGGGGTGCCTTAATGTCTGAAA

-70

45 ATTGGGGTATATAAAATATGGCGATTCTCCG

-50

50 GAGAATTTTCAGTTTCTTTCTTTCTTC

-10

55 CACTATTCTTTCTATAAACTATACTACA

10 ATGCCTTCTATTAAGTTGAACCTCTGGTTAC

30

50

GACATGCCAGCCGTGGTTCTGGCTGGTGG

70
AAAGTCGACGTGACACACCTGTTCTGAACAG

90

110
ATCTACCGTGCTATCAAGACCGGGTACAGA

10

130
TTGTTCGACGGTGCCGAAGATTACGCCAAC

150

15

170
GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG

190

20

210
GCCATTGACGAAGGTATCGTCAAGCGTGAA

230

25

250
AACTACCACCACCCAGACAACGTCGAAAAG

270

30

290
GCCTTGAACAGAACCCCTTCTGACTTGCAA

310

35

330
GTTGACTACGTTGACTTGTCTTGATCCAC

350

40

370
TTCCCAGTCACCTTCAAGTTGTTCCATTAA

390

45

410
GGTAAGGGTGACAACCTCGACTACGAAGAT

430

50

450
GTTCCAATTTAGAGACCTGGAAAGGCTCTT

470

490
GAAAAGTTGGTCAAGGCCGGTAAGATCAGA

490

5 TCTATCGGTGTTCTAACCTCCCAGGTGCT 510

530

10 TTGCTCTGGACTTGTTGAGAGGTGCTACC

550

15 ATCAAGCCATCTGTCTTGCAAGTTGAACAC 570

590

20 CACCCATACTGCAACAACCAAGATTGATC

610

25 GAATTCGCTCAATCCCGTGGTATTGCTGTC 630

650

30 ACCGCTTACTCTCGTTCGGTCCTCAATCT

670

35 TTCGTTGAATTGAACCAAGGTAGAGCTTG 690

710

40 AACACTTCTCCATTGTTCGAGAACGAAACT

730

45 ATCAAGGCTATCGCTGCTAACGCACGGTAAG 750

770

50 TCTCCAGCTCAAGTCTTGAGATGGTCT

790

55 TCCCAACAGGGCATTTGCCATCAATTCCAAAG 810

830

60 TCCAAACACTGTCCCAAGATTGTTGCCAAAC

850

65 AAGGACGTCAACAGCTCGACTTGGACGAA 870

890

70 CAAGATTCGCTGACATTGCCAAGTTGGAC

55

5

910 ATCAACTTGAGATTCAACGACCCATGGGAC 930

10 950 TGGGACAAGATTCTATCTTCGTCTAAGAA

15 970 GGTTGCTTATAGAGAGGAAATAAAACCTA 990

20 1010 ATATAACATTGATTGTACATTTAAAATTGAA

25 1030 1050 TATTGTAGCTAGCAGATTGGAAATTAAA

30 1070 ATGGGAAGGTGATTCTATCCGTACGAATGA

35 1090 1110 TCTCTATGTACATACACGTTGAAGATAGCA

40 1130 GTACAGTAGACATCAAGTCTACAGATCATT

45 1150 1170 AACATATCTTAAATTGTAGAAAACCTATAA

50 1190 ACTTTCAATTCAAACCATGTCTGCCAAGG

55 1210 1230 AATCAATTGAGATTTTTCGCAGCCAAAC

1250 TTGAATCCAAAAATAAAAAACGTCAATTGTC

1270 1290 TGAAACAACTCTATCTTATCTTCACCTCA

1310 TCAATTCAATTGCATATCATAAAAGCCTCCG

1330
ATAGCATACAAA¹³⁵⁰
ACTACTTCTGCATCATAT

1370
CTAAATCATAGTGC¹³⁹⁰
CATATTCAAGTAACAAT

1410
ACCGGTAAGAA¹⁴³⁰
ACTTCTATTTTTTAGTCT

1450
AAGATCAAACCC¹⁴⁷⁰
CTCCAGTTGTACAGTCAG

1490
TCATATA¹⁵¹⁰
TAGTGAACACC¹⁵³⁰
GTACAAATATGGTAT

1550
TCTGCCAAGTTGAGCA¹⁵⁷⁰
ACTTTAATTAGA

1590
GACAAAGTCGTCT¹⁶¹⁰
GTGATGTAGGCACC

1650
TCTGGAGTGC¹⁶³⁰
AAACATCTCCAGTTGTCAAA

1670
TATCAAACACTGACCAGGCTC¹⁶⁹⁰
AACTGGTA

GAAGATTTCGTTTCGGGATC

6. Procédé selon la revendication 2, dans lequel la séquence d'ADN comprend la séquence suivante de nucléotides :

-310
TCTAGACCACCCCTAACGTCGTCCCTATGTCG -290

5

-270
TATGTTGCCTACTACAAAGTTACTAGC

10

-250
AAATATCCGCAGCAACAAACAGCTGCCCTCT -230

15

-210
TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

20

-190
CGCTTTCGGGCTCCAGCTTCTGTCCCTCTGC -170

25

-150
GGCTGCTGCACATAACGCGGGGACAATGAC

30

-130
TTCTCCAGCTTTATTATAAAAAGGAGCCAT -110

35

-90
CTCCTCCAGGTGAAAAATTACGATCAAATT

-70
TTACTCTTCCATTGTCTCTGTGTATAAC -50

40

-30
TCACTTTAGTTGTTCAATCACCCCTAAT

45

-10
ACTCTTCACACAAATTAAATGACTGCTAAC 10

50

30
CCTTCCTTGGTGTGAACAAAGATCGACGAC

55
ATTTCGTTCGAAACTTACGATGCCCGAGAA 70

5 70 AAAGTCGACGTGACACCTGTTCTGAACAG
10 110 ATCTACCGTGCTATCAAGACCGGTTACAGA
15 130 150 TTGTTCGACGGTGCCGAAGATTACGCCAAC
20 170 GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG
25 190 210 GCCATTGACGAAGGTATCGTCAAGCGTGAA
30 230 GACTTGTTCCTTACCTCCAAGTTGTGGAAC
35 250 270 AACTACCACCAACCCAGACAACGTCGAAAAG
40 290 GCCTTGAACAGAACCCCTTCTGACTTGCAA
45 310 330 GTTGACTACGTTGACTTGTCTTGATCCAC
50 350 TTCCCCAGTCACCTTCAAGTTGTTCCATTA
55 370 390 GAAGAAAAGTACCCACCAAGGATTCTACTGT
60 410 GGTAAGGGTGACAACCTCGACTACGAAGAT
65 430 450 GTTCCAATTTAGAGACCTGGAAAGGCTCTT
70 470 GAAAAGTTGGTCAAGGCCGGTAAGATCAGA

90

ATCTCTGAACCTACCGATGTCCCTCGTCCAG

110 130

5 GTCAAGAAAACCGGTATCTGTGGTTCCGAC

150

10 ATCCACTTCTACGCCCATGGTAGAATCGGT

170 190

15 AACTTCGTTTGACCAAGCCAATGGTCTTG

210

20 GGTACCGAATCCGCCCGGTACTGTTGTCCAG

230 250

GTTGGTAAGGGTGTACCTCTCTTAAGGTT

270

25 GGTGACAACGTCGCTATCGAACCAAGGTATT

290 310

30 CCATCCAGATTCTCCGACGAATAACAAGAGC

330

35 GGTCACTACAACTTGTGTCCCTCACATGGCC

350 370

40 TTCGCCGCTACTCCTAACTCCAAGGAAGGC

390

45 GAACCAAACCCACCAGGTACCTTATGTAAG

410 430

TACTTCAAGTCGCCAGAAGACTTCTGGTC

450

50 AAGTTGCCAGACCACGTCAGCTTGGAACTC

470 490
GGTGCTCTGTTGAGCCATTGTCTGTTGGT

510
GTCCACGCCCTCCAAGTTGGGTTCCGTTGCT

530 550
TTCGGCGACTACGTTGCCGTCTTGGTGCT

570
GGTCCTGTTGGTCTTTGGCTGCTGCTGTC

590 610
GCCAAGACCTTCGGTGCTAACGGTGTCACTC

630
GTCGTTGACATTTCGACAACAAGTTGAAG

650 670
ATGGCCAAGGACATTGGTGCTGCTACTCAC

690
ACCTTCAACTCCAAGACCGGTGGTTCTGAA

710 730
GAAATTGATCCTAGGCTTCGGTGGTAAACGTG

750
CCAAACGGCGTTCTGGAAATGTACTGGTGCCT

770 790
GAAACCTTGTNTCAAGTGGGTGTTGACGCC

810
ATTGCCCCAGGTGGTCGTTCGTTCAAGTT

830 850
GGTAACGCTGCTGGTCCAGTCAGCTTCCCA

870

ATCACCGTTTCGCCATGAAGGAATTGACT

890 910

5 TTGTTCGGTTCTTCAGATAACGGATTCAAC

10 930

GACTACAAGACTGCTGTTGGAATCTTGAC

15 950 970

ACTAACTACCAAAACGGTAGAGAAAATGCT

20 990

CCAATTGACTTTGAACAAATTGATCACCCAC

25 1010 1030

AGATAACAAGTTCAAGGACGCTATTGAAGCC

30 1050

TACCGACTTGGTCAGAGCCGGTAAGGGTGCT

35 1070 1090

GTCAAAGTGTCTCATTGACGGCCCTGAGTAA

40 1110

GTCAACCGCTTGGCTGGCCCTAAGTGAACC

45 1130 1150

AGAACCGAAATGTTATGAAATAGCTTTA

50 1170

TAGACCTTATCGAAATTTATGAAACTAA

55 1190 1210

TAGAAAAGACAGTGTAGAAGTTATATGGTT

55 1230

GCATCACGTGAGTTCTTGAATTCTGAAA

1250 1270
GTGAAGTCTGGTCGAAACAAACAAACAAA

5 1290
AAAATATTTCAGCAAGAGTTGATTCTTT

10 1310 1330
TCTGGAGATTTGGTAATTGACAGAGAACCC

15 1350
CCTTTCTGCTATTGCCATCTAACACATCCTT

20 1370 1390
GAATAGAACTTTACTGGATGGCCGCCTAGT

25 1410
GTTGAGTATATATTATCAACCAAAATCCTG

30 1430 1450
TATATAGTCTCTGAAAAAATTGACTATCCT

35 1470
AACCTAACAAAAGAGCACCATATAATGCAAGC

40 1490 1510
TCATAGTTCTTAGAGACAÇCAAQTATACTT

45 1530
AGCCAAACAAAATGTCCTTGGCCTCTAAAG

50 1550 1570
AAGCATTCTCGCAAGCTTCCCCAGAGTTGC

55 1590
ACAACTTCTCATCAAGTTACCCCCAGAC

60 1610 1630
CGTTTGCCGAAATATTCGGAAAGCCTTCGA

65 CTATAGTGGATCC

55 7. Procédé de préparation d'une combinaison de séquences d'ADN, ledit procédé comprenant la combinaison d'une première séquence d'ADN susceptible d'être obtenue selon l'une quelconque des revendications 1 à 6 et une ou plusieurs autres séquences d'ADN capables de réguler l'expression d'un gène d' structure encodé par ladit séquence d'ADN dans un micro-organisme hôte d'une manière connue en soi.

8. Procédé selon la revendication 7, dans lequel ladite combinaison de séquences comprend des modifications des séquences d'ADN conservant leur capacité à exprimer une enzyme fonctionnelle ayant une activité de réductase du xylose ou de déshydrogénase du xylitol.

5 9. Procédé selon l'une quelconque des revendications 7 ou 8, dans lequel ledit gène de structure contient des séquences d'ADN dérivées du gène de structure codant pour la réductase du xylose ou la déshydrogénase du xylitol qui modifie ledit produit de protéine tout en conservant ses fonctions d'une façon telle que ledit produit de protéine est exprimé comme un produit de gène ayant une activité enzymatique.

10 10. Procédé selon l'une quelconque des revendications 7 à 9, dans lequel lesdites séquences d'ADN capables de réguler l'expression dudit gène de structure dans un micro-organisme hôte, sont dérivées dudit micro-organisme hôte.

15 11. Procédé selon la revendication 10, dans lequel lesdites séquences d'ADN capables de réguler l'expression sont des promoteurs susceptibles d'être induits.

12. Procédé selon la revendication 11, caractérisé en ce que lesdites séquences d'ADN capables de réguler l'expression sont choisies parmi les promoteurs suivants :
ADH1, ADH2, PDC, GAL1/10.

20 13. Procédé selon l'une quelconque des revendications 10 à 12, dans lequel ladite séquence d'ADN capable de réguler l'expression dudit gène de structure, est un promoteur fort, conduisant à une surexpression de la protéine encodée par ledit gène de structure.

14. Procédé de préparation d'un vecteur, ledit procédé comprenant l'insertion d'une séquence d'ADN susceptible d'être obtenue selon l'une quelconque des revendications 1 à 6 ou d'une combinaison de séquences d'ADN susceptibles d'être obtenues selon l'une quelconque des revendications 7 à 13, dans un plasmide hôte.

25 15. Procédé selon la revendication 14, caractérisé en ce qu'il produit un vecteur choisi parmi le groupe comprenant les plasmides pR1, pR2, pD1, pD2, pRD1, pXRa, pXRb, pXDH, pXR, pXDH-HIS3, pXR-LEU2.

16. Procédé de préparation d'un micro-organisme capable d'exprimer une réductase du xylose ou une réductase du xylose et une deshydrogénase du xylitol, dans lequel des séquences d'ADN comprenant les séquences d'ADN susceptibles d'être obtenues selon l'une quelconque des revendications 1 à 6 ou une combinaison de séquences d'ADN susceptibles d'être obtenues selon l'une quelconque des revendications 7 à 13, codant pour ladite réductase du xylose ou ladite réductase du xylose et ladite deshydrogénase du xylitol, sont introduites dans un micro-organisme hôte.

30 17. Procédé selon la revendication 16, caractérisé en ce que ledit micro-organisme hôte est choisi parmi un groupe constitué de levures des genres *Saccharomyces*, *Schizosaccharomyces*, *Schwanniomyces*, *Kluyveromyces*, *Pichia*, *Hansenula*, *Candida*, *Debaryomyces*, *Metschnikowia*, *Pachysolen* ou *Paecilomyces* ou de bactéries du genre *Zymomonas*.

18. Procédé selon la revendication 17, caractérisé en ce que ledit micro-organisme est *Saccharomyces cerevisiae*.

45 19. Procédé selon la revendication 17, caractérisé en ce que ledit micro-organisme est *Schizosaccharomyces pombe*.

20. Procédé selon l'une quelconque des revendications 16 à 19, caractérisé en ce que ladite séquence d'ADN ou la combinaison de séquences d'ADN est intégrée dans le génome dudit micro-organisme.

50 21. Procédé selon l'une quelconque des revendications 17 à 21, caractérisé en ce que ledit micro-organisme est utile dans la production de biomasse, dans l'industrie alimentaire et dans les procédés de fermentation.

22. Procédé selon la revendication 21, caractérisé en ce que ledit micro-organisme est utile pour la fermentation du xylose dans l'éthanol.

55 23. Procédé de production de la réductase du xylose ou de la réductase du xylose et de la déshydrogénase du xylitol par culture d'un micro-organisme susceptible d'être obtenu selon l'une quelconque des revendications 16 à 20

sous des conditions appropriées et récupération du(des)dits enzyme(s) d'une manière connue en soi.

24. Procédé selon la revendication 23, caractérisé en ce que ledit micro-organisme est choisi pour la fermentation efficace du xylulose.

5

25. Procédé selon la revendication 23 ou 24, caractérisé en ce que ledit micro-organisme a reçu lesdites séquences d'ADN ou ladite combinaison de séquences d'ADN par transformation utilisant un vecteur, ledit vecteur étant de préférence un fragment d'ADN ou un plasmide.

10

26. Procédé selon la revendication 25, caractérisé en ce que ledit vecteur contient de l'ADN, qui est homologue à l'ADN dudit micro-organisme, conduisant à l'intégration dans le génome dudit micro-organisme.

27. Procédé de fabrication d'éthanol, caractérisé en ce qu'on utilise un micro-organisme selon l'une quelconque des revendications 16 à 22.

15

28. Procédé selon la revendication 27, caractérisé en ce que le procédé de fermentation est adapté à la production de boissons alcoolisées ou d'une protéine de cellule unique produite à partir de substrats contenant du xylose libre, de préférence libéré par l'activité de la xylanase et/ou de la xylosidase.

20

29. Procédé pour la production de biomasse, caractérisé en ce que l'on utilise le micro-organisme hôte selon l'une quelconque des revendications 16 à 22.

25

30

35

40

45

50

55

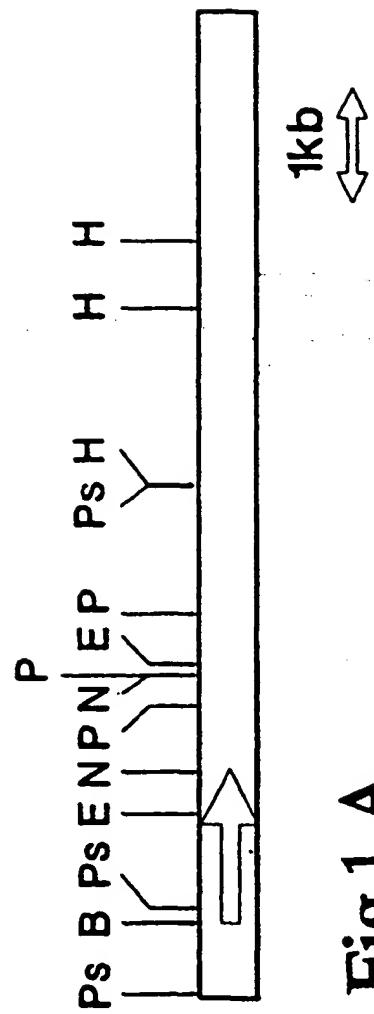


Fig.1 A

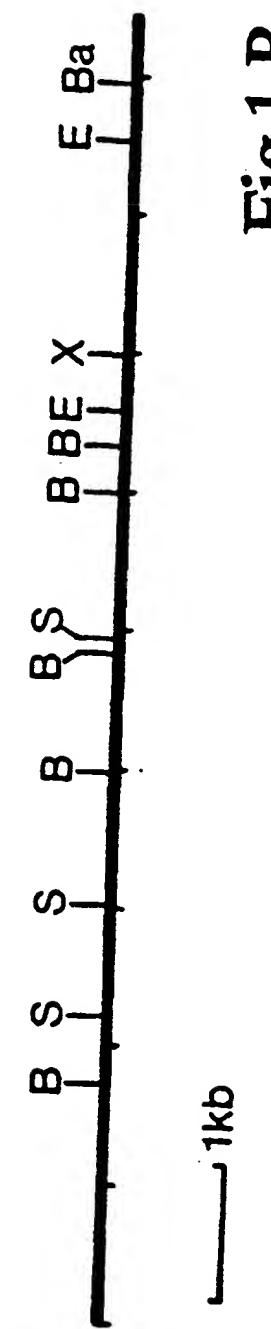


Fig.1 B

Fig.2A (1)

-350
GGATCCACAGACACTAATTGGTTCTA

-310
CATTATT CGT GTTCAGACACAAACCC CAGC

-290
GTTGGCGGTTCTGTCTCGT CCTCCAGC

-250
ACCTTCTTGCTCAACCCCAGAAGGTGCACA

-230
CTGCAGACACACATA CATACAGAGAACCTGG

-190
AACAAATATCGGTGT CGGTGACCGAAATGT

-170
GCAAACCCAGACACGACTAATAAACCTGGC

-130
AGCTCCAATACCGCCGACAACAGGTGAGGT

-110
GACCGATGGGGTGCCAATTATGTCTGAAA

-70
ATTGGGGTATATAAATATGGCGATTCTCCG

-50
GAGAATTTCAGTTCTTTCATTTCTC

-10
CAGTATTCTTTCTATAACA ACTATACTACA

10	30
ATGCCTTCTATTAAGTTGA	ACTCTGGTTAC
M P S I K L N S G Y	

Fig.2A (2)

50
 GACATGCCAGCCGTCGGTTCTGGCTGTTGG
 D M P A V G F G C W

70 90
 AAAGTCGACGTGACACACTGTTCTGAACAG
 K V D V D T C S E Q

110
 ATCTACCGTGCTATCAAGACC GGTTACAGA
 I Y R A I K T G Y R

130 150
 TTGTTCGACGGTGC CGAAGATTACGCCAAC
 L F D G A E D Y A N

170
 GAAAAGTTAGTTGGTGCCGGTGTCAAGAAG
 E K L V G A G V K K

190 210
 GCCATTGACGAAGGTATCGTCAAGCGTGAA
 A I D E G I V K R E

230
 GACTTGTTCCTTACCTCCAAGTTGTGGAAC
 D L F L T S K L W N

250 270
 AACTACCACCA CCCAGACAACGTCGAAAAG
 N Y H H P D N V E K

290
 GCCTTGAACAGAACCCCTTCTGACTTGCAA
 A L N R T L S D L Q

310 330
 GTTGACTACGTTGACTTGTTCTGATCCAC
 V D Y V D L F L I H

Fig.2A (3)

350

TTCCCAGTCACCTCAAGTTCGTTCCATT
F P V T F K F V P L

370 390

GAAGAAAAGTACCCACCAGGATTCTACTGT
E E K Y P P G F Y C

410

GGTAAGGGTGACAACCTCGACTACGAAGAT
G K G D N F D Y E D

430 450

GTTCCAATTTAGAGACCTGGAAGGCTCTT
V P I L E T W K A L

470

GAAAAGTTGGTCAAGGCCGGTAAGATCAGA
E K L V K A G K I R

490 510

TCTATCGGTGTTCTAACTTCCCAGGTGCT
S I G V S N F P G A

530

TTGCTCTTGGACTTGTTGAGAGGTGCTACC
L L L D L L R G A T

550 570

ATCAAGCCATCTGTCTGCAAGTTAACAC
I K P S V L Q V E H

590

CACCCATACTTGCAACAACCAAGATTGATC
H P Y L Q Q P R L I

610 630

GAATTGCTCAATCCCGTGGTATTGCTGTC
E F A Q S R G I A V

Fig.2A (4)

650
 ACCGCTTACTCTCGTTGGTCCTCAATCT
 T A Y S S F G P Q S

670 690
 TTCGTTGAATTGAACCAAGGTAGAGCTTG
 F V E L N Q G R A L

710
 AACACTTCTCCATTGTTGAGAACGAAACT
 N T S P L F E N E T

730 750
 ATCAAGGCTATCGCTGCTAACGACGGTAAG
 I K A I A A K H G K

770
 TCTCCAGCTCAAGTCTTGTGAGATGGTCT
 S P A Q V L L R W S

790 810
 TCCCAAAGAGGCATTGCCATCATTCAAAG
 S Q R G I A I I P K

830
 TCCAACACTGTCCAAGATTGTTGGAAAAC
 S N T V P R L L E N

850 870
 AAGGACGTCAACAGCTCGACTTGGACGAA
 K D V N S F D L D E

890
 CAAGATTGCTGACATTGCCAAGTTGGAC
 Q D F A D I A K L D

910 930
 ATCAAACATTGAGATTCAACGACCCATGGGAC
 I N L R F N D P W D

Fig.2A (5)

950
 TGGGACAAGATTCTATCTTCGTCTAAGAA
 W D K I P I F V *

970 990
 GGTTGCTTATAGAGAGGAAATAAAACCTA

1010
 ATATACATTGATTGTACATTTAAAATTGAA

1030 1050
 TATTGTAGCTAGCAGATTCGGAAATTAAA

1070
 ATGGGAAGGTGATTCTATCCGTACGAATGA

1090 1110
 TCTCTATGTACATACACGTTGAAGATAGCA

1130
 GTACAGTAGACATCAAGTCTACAGATCATT

1150 1170
 AACACATATCTTAAATTGTAGAAAATATAA

1190
 ACTTTCAATTCAAACCATGTCTGCCAAGG

1210 1230
 AATCAAATGAGATTTTCGCAGCCAAAC

1250
 TTGAATCCAAAAATAAAAACGTCTTGTC

1270 1290
 TGAAACAACTCTATCTTATCTTCACCTCA

1310
 TCAATTGCATATCATAAAAGCCTCCG

Fig.2A (6)

1330 1350
ATAGCATACAAAACACTACTTCTGCATCATAT

 1370
CTAAATCATAGTGCCATATTCAAGTAACAAT

 1390 1410
ACCGGTAAGAAACCTCTATTTTTTAGTCT

 1430
GCCTTAACGAGATGCAGATCGATGCAACGT

 1450 1470
AAGATCAAACCCCTCCAGTTGTACAGTCAG

 1490
TCATATAGTGAACACCGTACAATATGGTAT

 1510 1530
CTACGTTCAAATAGACTCCAATACAGCTGG

 1550
TCTGCCAAGTTGAGCAACTTTAATTAGA

 1570 1590
GACAAAGTCGTCTCTGTTGATGTAGGCACC

 1610
ACACATTCTCTCTGCCGTGAACCTCTGT

 1630 1650
TCTGGAGTGGAAACATCTCCAGTTGTAAA

 1670
TATCAAACACTGACCAGGCTTCAACTGGTA

 1690
GAAGATTTCGTTTCGGGATCC

Fig.2B (1)

-310 -290
 TCTAGACCACCCCTAACAGTCGTCCCTATGTCG

-270
 TATGTTGCCTCTACTACAAAGTTACTAGC

-250 -230
 AAATATCCGCAGCAACAAACAGCTGCCCTCT

-210
 TCCAGCTTCTTAGTGTGTTGGCCGAAAAGG

-190 -170
 CGCTTTCGGGCTCCAGCTCTGTCCCTCTGC

-150
 GGCTGCTGCACATAACGCGGGGACAATGAC

-130 -110
 TTCTCCAGCTTTATTATAAAAGGAGCCAT

-90
 CTCCTCCAGGTGAAAAATTACGATCAACTT

-70 -50
 TTACTCTTTCCATTGTCTCTGTGTATAC

-30
 TCACTTTAGTTGTTCAATCACCCCTAAT

-10 10
 ACTCTTCACACAATTAAAATGACTGCTAAC
 M T A N

30
 CCTTCCTTGGTGTGAACAAAGATCGACGAC
 P S L V L N K I D D

Fig.2B (2)

50 ATTCGTTCGAAACTTACGATGCCAGAA
 I S F E T Y D A P E
 90 ATCTCTGAACCTACCGATGTCCTCGTCCAG
 I S E P T D V L V Q
 110 GTCAAGAAAACCGGTATCTGTGGTTCCGAC
 V K K T G I C G S D
 150 ATCCACTTCTACGCCATGGTAGAATCGGT
 I H F Y A H G R I G
 170 AACTCGTTTGACCAAGCCAATGGTCTTG
 N F V L T K P M V L
 210 GGTACCGAATCCGCCGGTACTGTTGTCCAG
 G H E S A G T V V Q
 230 GTTGGTAAGGGTGTCACCTCTCTTAAGGTT
 V G K G V T S L K V
 270 GGTGACAACGTCGCTATCGAACCAAGGTATT
 G D N V A I E P G I
 290 CCATCCAGATTCTCCGACGAATACAAGAGC
 P S R F S D E Y K S
 330 GGTCACTACAACCTGTGTCTCACATGGCC
 G H Y N L C P H M A

Fig.2B (3)

350 370

TTCGCCGCTACTCCTAACTCCAAGGAAGGC
F A A T P N S K E G

390

GAACCAAACCCACCAGGTACCTTATGTAAG
E P N P P G T L C K

410 430

TACTTCAAGTCGCCAGAAGACTTCTGGTC
Y F K S P E D F L V

450

AAGTTGCCAGACCACGTCAGCTTGAACTC
K L P D H V S L E L

470 490

GGTGCTCTTGTGAGCCATTGTCTGTTGGT
G A L V E P L S V G

510

GTCCACGCCTCCAAGTTGGGTTCCGTTGCT
V H A S K L G S V A

530 550

TTCGGCGACTACGTTGCCGTCTTGGTGCT
F G D Y V A V F G A

570

GGTCCTGTTGGTCTTTGGCTGCTGCTGTC
G P V G L L A A A V

590 610

GCCAAGACCTTCGGTGCTAACGGGTGTCATC
A K T F G A K G V I

630

GTCGTTGACATTTCGACAACAAGTTGAAG
V V D I F D N K L K

Fig.2B (4)

650	670
ATGGCCAAGGACATTGGTGCTGCTACTCAC	
M A K D I G A A T H	
690	710
ACCTTCAACTCCAAGACCGGGTGGTTCTGAA	
T F N S K T G G S E	
730	750
GAATTGATCAAGGCTTCGGTGGTAACGTG	
E L I K A F G G N V	
770	790
CCAAACGTCGTTTGGAAATGTACTGGTGCT	
P N V V L E C T G A	
810	830
GAACCTTGTATCAAGTTGGGTGTTGACGCC	
E P C I K L G V D A	
850	870
ATTGCCCCAGGTGGTCGTTCTGTTCAAGTT	
I A P G G R F V Q V	
890	910
GGTAACGCTGCTGGTCCAGTCAGCTTCCCA	
G N A A G P V S F P	
930	950
ATCACCGTTTCGCCATGAAGGAATTGACT	
I T V F A M K E L T	
970	990
TTGTTGGTTCTTCAGATACTGGATTCAAC	
L F G S F R Y G F N	
1010	1030
GAATACAAAGACTGCTGTTGGAATCTTGAC	
D Y K T A V G I F D	

Fig.2B (5)

950 970
 ACTAACTACCAAAACGGTAGAGAAAATGCT
 T N Y Q N G R E N A
 990
 CCAATTGACTTTGAACAATTGATCACCCAC
 P I D F E Q L I T H
 1010 1030
 AGATACAAAGTTCAAGGACGCTATTGAAGCC
 R Y K F K D A I E A
 1050
 TACGACTTGGTCAGAGCCGGTAAGGGTGCT
 Y D L V R A G K G A
 1070 1090
 GTCAAGTGTCTCATGACGGCCCTGAGTAA
 V K C L I D G P E *
 1110
 GTCAAACCGCTTGGCTGGCCCCAAAGTGAACC
 1130 1150
 AGAAAACGAAAATGATTATCAAATAGCTTA
 1170
 TAGACCTTATCGAAATTTATGTAAACTAA
 1190 1210
 TAGAAAAGACAGTGTAGAAGTTATATGGTT
 1230
 GCATCACGTGAGTTCTTGAATTCTTGAAAA
 1250 1270
 GTGAAGTCTTGGTCGGAACAAACAAACAAA
 1290
 AAAATATTTCAGCAAGAGTTGATTCTT

Fig.2B (6)

1310 1330
 TCTGGAGATTTGGTAATTGACAGAGAAC

 1350
 CCTTTCTGCTATTGCCATCTAACACATCCTT

 1370 1390
 GAATAGAACTTTACTGGATGGCCGCCTAGT

 1410
 GTTGAGTATATATTATCAACCAAAATCCTG

 1430 1450
 TATATAGTCTCTGAAAAATTGACTATCCT

 1470
 AACTTAACAAAAGAGCACCATATAATGCAAGC

 1490 1510
 TCATAGTTCTTAGAGACACCAACTATACTT

 1530
 AGCCAAACAAAATGTCCCTGGCCTCTAAAG

 1550 1570
 AAGCATTCAAGCTTCCCCAGAAGTTGC

 1590
 ACAACTTCTTCATCAAGTTACCCCCAGAC

 1610 1630
 CGTTTGCAGAATATTGGAAAAGCCTTCGA

CTATAGTGGATCC

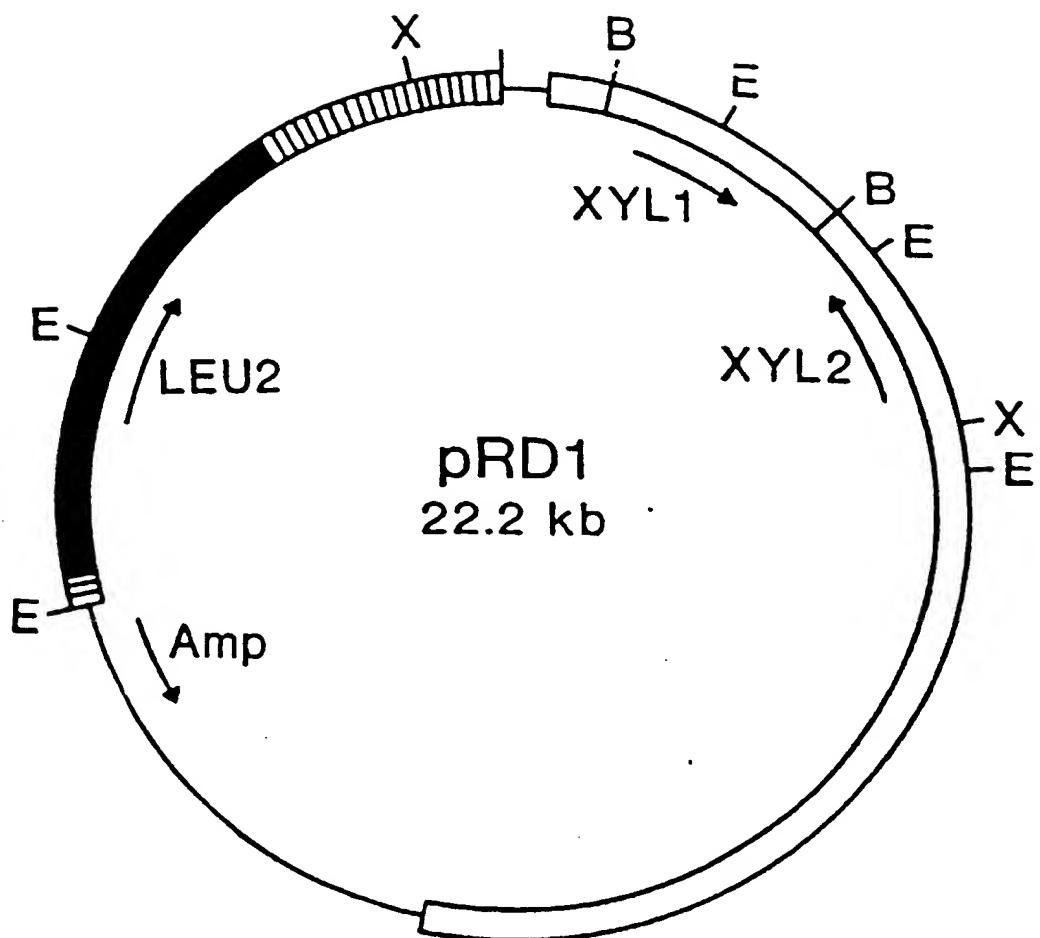


Fig.3

- *P. stipitis*
- *S. cerevisiae*
- 2μ
- pBR322

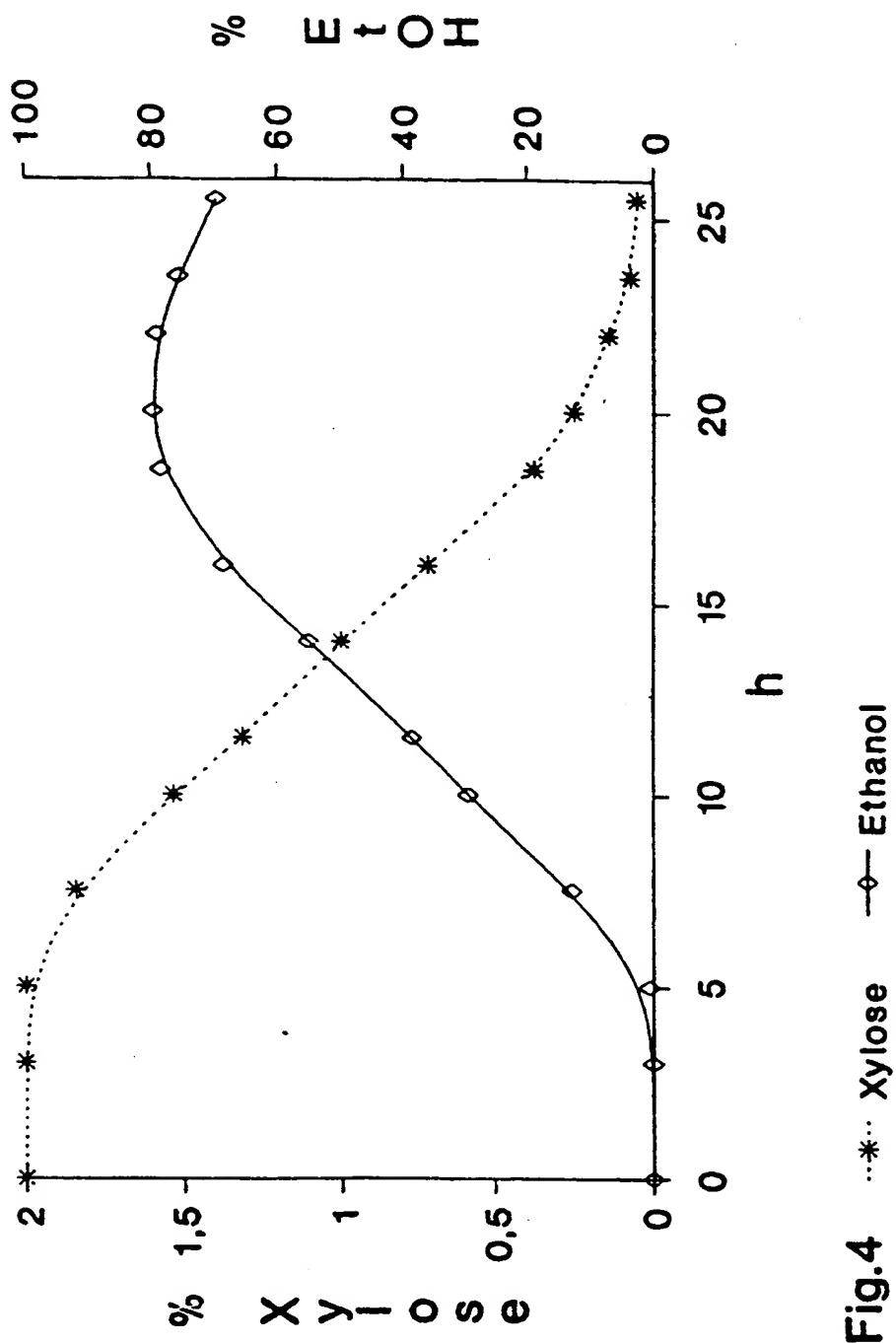


Fig.4 ...*... Xylose —◇— Ethanol

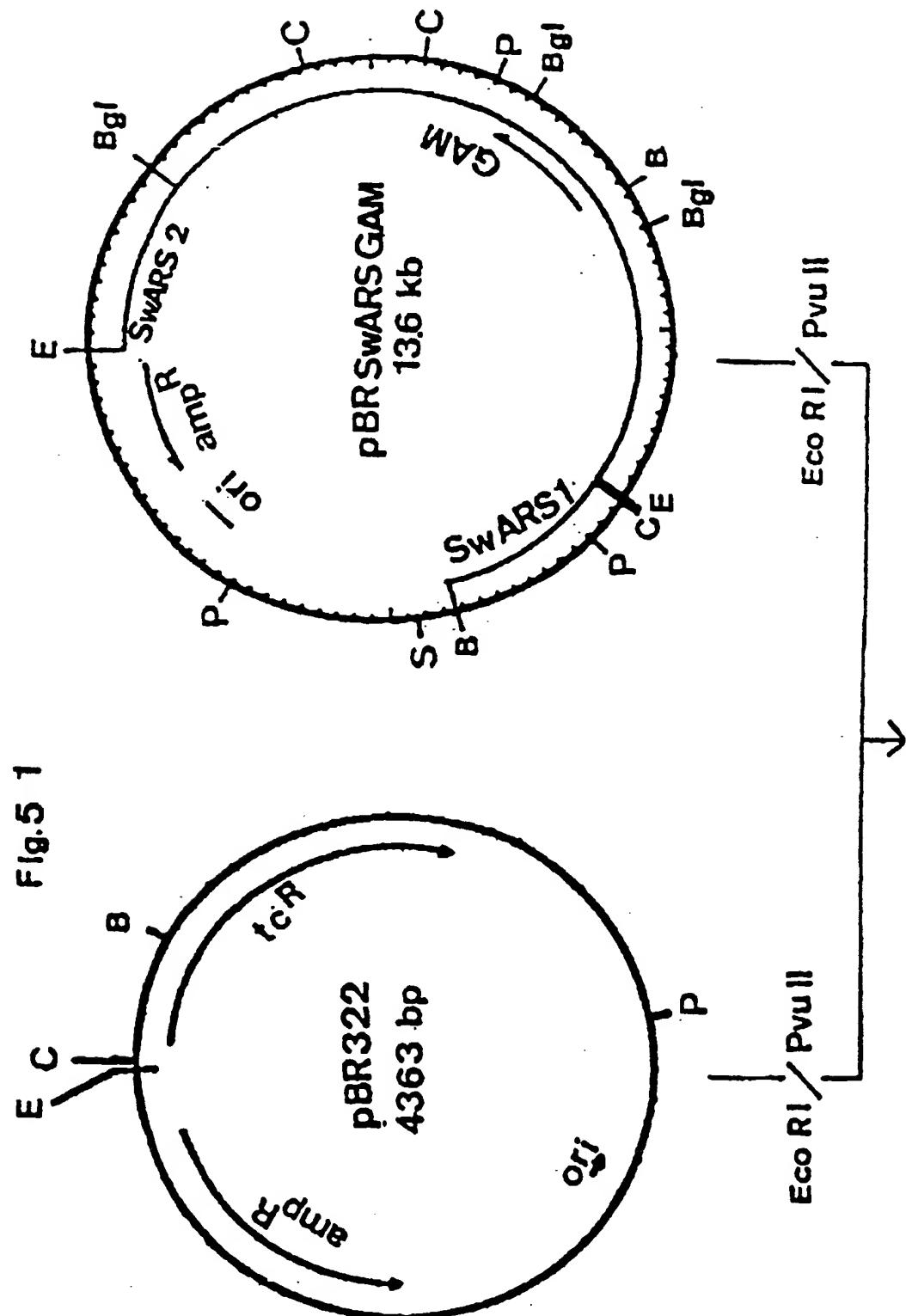
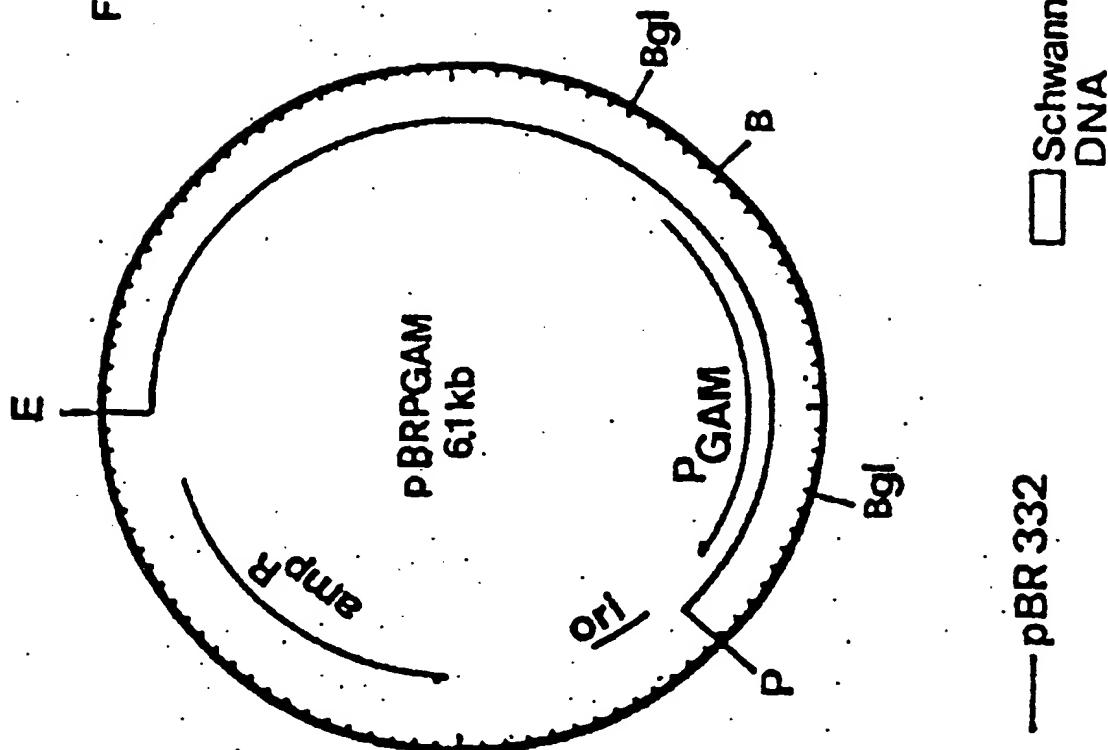


Fig. 5 1

Fig.5 2



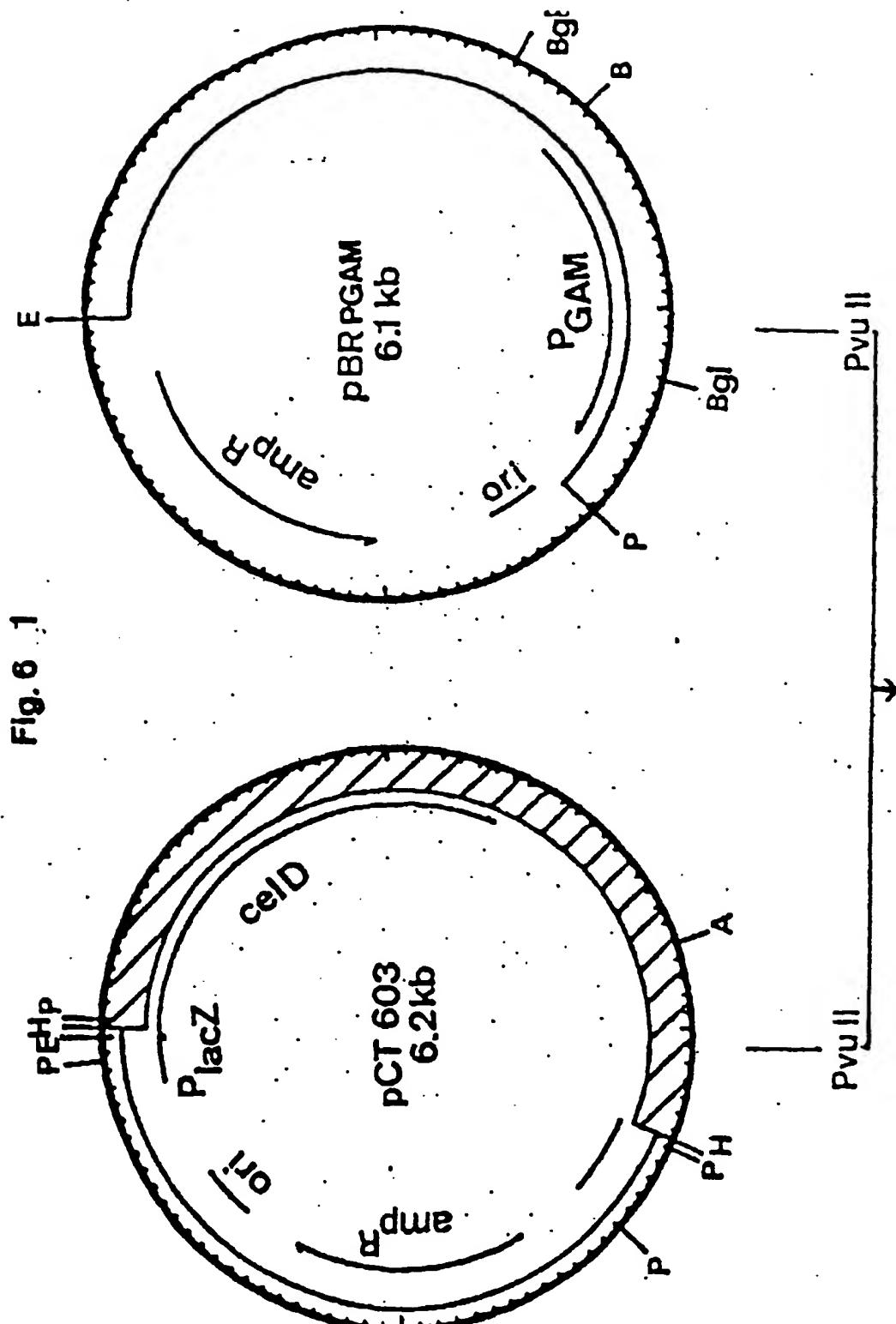
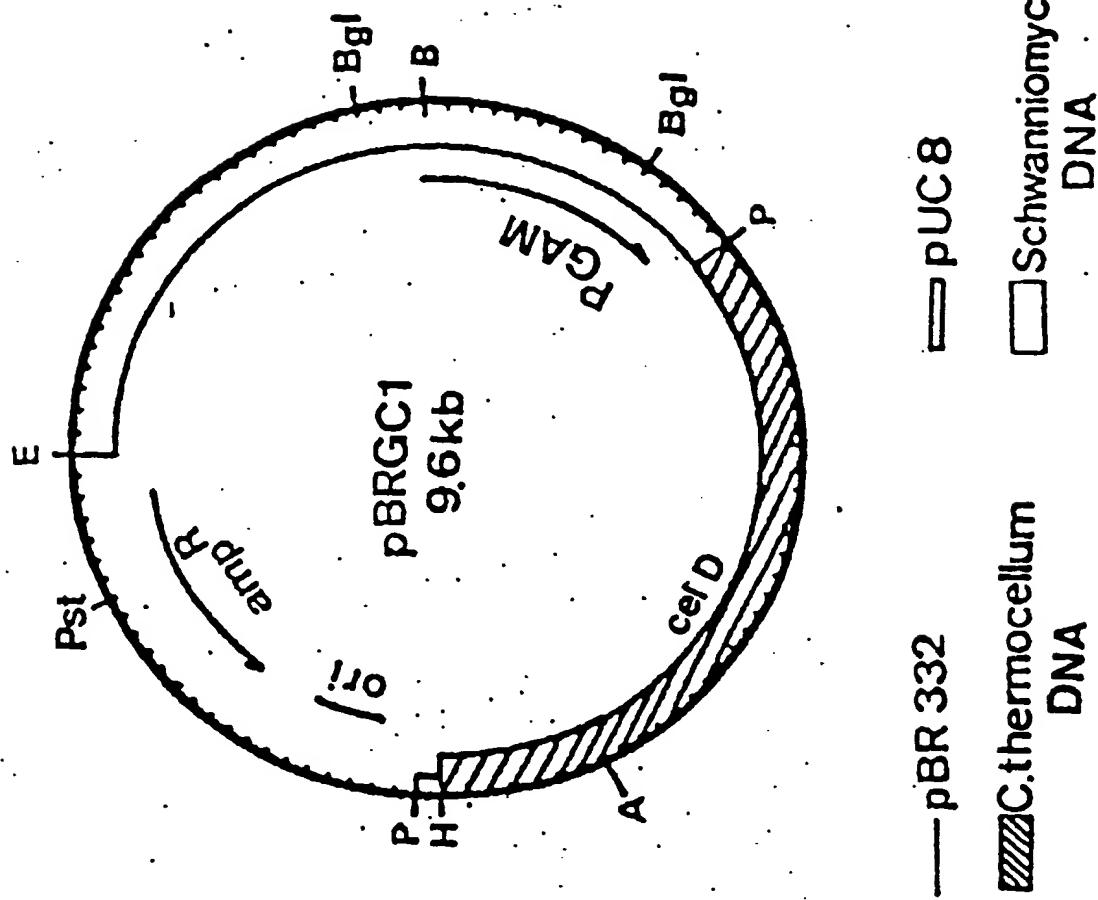
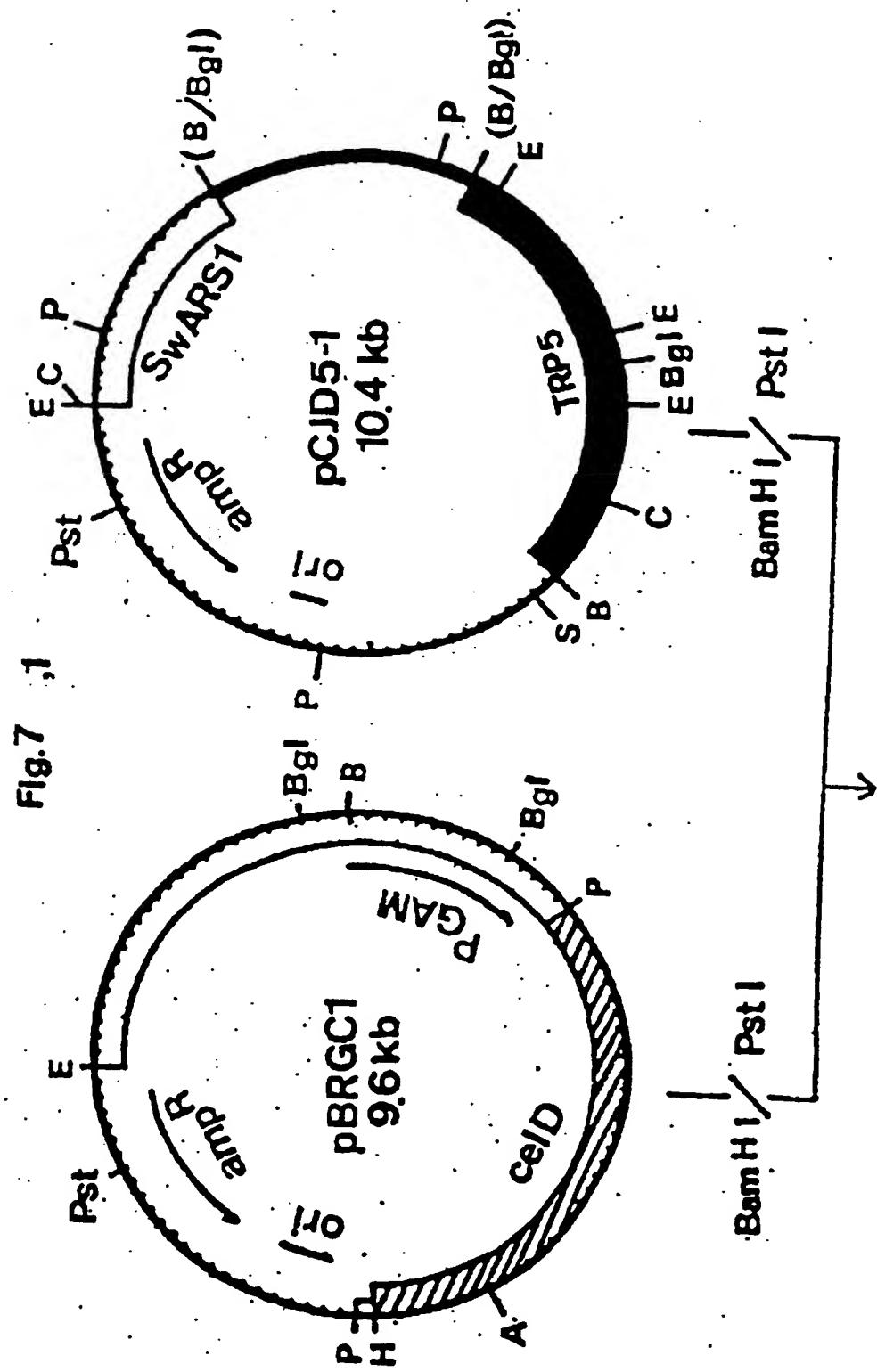


Fig. 6 ,2





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